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(54) Title: VACCINE COMPOSITION

(57) Abstract: The present invention relates to the field of Gram-negative bacterial vaccine compositions, their manufacture, and the use of such compositions in medicine. More particularly it relates to the field of useful Gram-negative bacterial outer membrane vesicle (or bleb) compositions comprising heterologously expressed *Chlamydia* antigens, and advantageous methods of rendering these compositions more effective and safer as a vaccine.

VACCINE COMPOSITION

FIELD OF THE INVENTION

The present invention relates to the field of Gram-negative bacterial vaccine compositions, their manufacture, and the use of such compositions in medicine. More particularly it relates to the field of useful Gram-negative bacterial outer membrane vesicle (or bleb) compositions comprising heterologously expressed *Chlamydia* antigens, and advantageous methods of rendering these compositions more effective and safer as a vaccine.

BACKGROUND OF THE INVENTION

Chlamydiae are obligate intracellular Gram negative bacteria which replicate only in cytoplasmic inclusions of eukaryotic cells. They have a unique developmental cycle which is represented by two major forms, the spore-like elementary body (EB) which is the infectious form transmitted from cell to cell, and the non infectious, metabolically active reticulate body (RB) which replicates within the host-cell.

Of the four known chlamydial species, *Chlamydia trachomatis* and *C. pneumoniae* are the important human pathogens. The recently defined species *C. pneumoniae* (Grayston 1989) is now recognized as a major cause of respiratory tract infections (Grayston 1993) and data are now growing for an association with atherosclerosis. The association is supported by seroepidemiological studies, studies demonstrating the presence of the bacterium in the atherosclerotic lesions, studies showing *C. pneumoniae* capability to replicate in the different cell types present in the atheroclerotic lesions, interventional trials with antibiotics in patients with coronary artery disease and experimental respiratory tract infection in rabbits or apolipoprotein-E deficient mice which leads to inflammatory changes in the aorta (Danesh 1997, Fong 1997, Laitinen 1997, Moazed 1997). Overall, those data implicate *C. pneumoniae* as a causative and/or aggravating factor of atherosclerosis.

C. trachomatis is a major human pathogen; transmitted from human to human (there is no known animal reservoir), it causes ocular and genital infections which can result in long term sequelae. Trachoma, a chlamydial ocular infection, is endemic in several developing countries and is the world's leading cause of preventable blindness with millions people affected by the disease. Genital chlamydial infections constitute

the most common bacterial sexually transmitted disease (STD) worldwide. In 1996, WHO generated a new set of global estimates for four major STDs drawing an extensive review of the published and unpublished prevalence data (Gerbase 1998). It has been estimated that in 1995, 4 and 5.2 million new cases of *C. trachomatis* infection occured in individuals aged 15-49 for North America and Western Europe, respectively; worlwide, *C. trachomatis* totalized an estimate of 89.1 million new cases. Collectively, data show higher infection rates in women as compared to men (Washington 1987, Peeling 1995, Cates 1991); higher incidence is found in adolescent and young adults, approximately 70% of the chlamydial infections being reported in the 15-24 years of age group (Peeling 1995).

There is a clear need for effective vaccines against *Chlamydia trachomatis* and *Chlamydia pneumoniae.*

Outer membrane vesicles (blebs)

Gram-negative bacteria are separated from the external medium by two successive layers of membrane structures. These structures, referred to as the cytoplasmic membrane and the outer membrane (OM), differ both structurally and functionally. The outer membrane plays an important role in the interaction of pathogenic bacteria with their respective hosts. Consequently, the surface exposed bacterial molecules represent important targets for the host immune response, making outer-membrane components attractive candidates in providing vaccine, diagnostic and therapeutics reagents.

Whole cell bacterial vaccines (killed or attenuated) have the advantage of supplying multiple antigens in their natural micro-environment. Drawbacks around this approach are the side effects induced by bacterial components such as endotoxin and peptidoglycan fragments. On the other hand, acellular subunit vaccines containing purified components from the outer membrane may supply only limited protection and may not present the antigens properly to the immune system of the host.

Proteins, phospholipids and lipopolysaccharides are the three major constituents found in the outer-membrane of all Gram-negative bacteria. These molecules are distributed asymmetrically: membrane phospholipids (mostly in the inner leaflet), lipooligosaccharides (exclusively in the outer leaflet) and proteins (inner and outer leaflet lipoproteins, integral or polytopic membrane proteins). For many

bacterial pathogens which impact on human health, lipopolysaccharide and outer-membrane proteins have been shown to be immunogenic and amenable to confer protection against the corresponding disease by way of immunization.

The OM of Gram-negative bacteria is dynamic and, depending on the environmental conditions, can undergo drastic morphological transformations. Among these manifestations, the formation of outer-membrane vesicles or "blebs" has been studied and documented in many Gram-negative bacteria (Zhou, L *et al.* 1998. *FEMS Microbiol. Lett.* 163: 223-228). Among these, a non-exhaustive list of bacterial pathogens reported to produce blebs include: *Bordetella pertussis*, *Borrelia burgdorferi*, *Brucella melitensis*, *Brucella ovis*, *Chlamydia psittaci*, *Chlamydia trachomatis*, *Esherichia coli*, *Haemophilus influenzae*, *Legionella pneumophila*, *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *Pseudomonas aeruginosa* and *Yersinia enterocolitica*. Although the biochemical mechanism responsible for the production of OM blebs is not fully understood, these outer membrane vesicles have been extensively studied as they represent a powerful methodology in order to isolate outer-membrane protein preparations in their native conformation.

Examples of bacterial species from which bleb vaccines can be made have been reviewed in WO 01/09350 (incorporated by reference herein). For example, *N. meningitidis* serogroup B (menB) excretes outer membrane blebs in sufficient quantities to allow their manufacture on an industrial scale. Such multicomponent outer-membrane protein vaccines from naturally-occurring menB strains have been found to be efficacious in protecting teenagers from menB disease and have become registered in Latin America. An alternative method of preparing outer-membrane vesicles is via the process of detergent extraction of the bacterial cells (EP 11243).

SUMMARY OF THE INVENTION

The present inventors have found that Gram-negative bacterial blebs are an ideal context to present *Chlamydia* outer membrane proteins. In particular gonococcal blebs are useful in the case of presenting *C. trachomatis* OMPs and meningococcal blebs are useful in the case of presenting *C. pneumoniae* OMPs. This is because a) these outer-membrane proteins can integrate into such blebs in a native (or near-native) conformation thus retaining a useful immunological effect; b) blebs

(particularly from *Neisseria* strains) can be produced in industrial quantities, c) blebs may be mucosally administered, and d) the combination of *Chlamydia* antigens with native bleb antigens can have important interactions for certain conditions such as salpingitis.

The present invention thus provides advantageous Gram-negative bacterial bleb preparations (derived from bleb-producing bacterial strains listed above, and preferably not derived from Chlamydia) presenting on its surface one or more recombinant (and preferably heterologous) protein antigens from *Chlamydia trachomatis* or *Chlamydia pneumoniae*. Advantagous vaccine formulations and methods of administration are also provided.

DESCRIPTION OF THE INVENTION

The present invention provides a Gram-negative bacterial bleb presenting on its surface one or more outer membrane protein from *Chlamydia*.

In the context of this application the term "presenting on its surface" indicates that the *Chlamydia* protein should be exposed to the outer surface of the bleb and tethered to the outer membrane (preferably by being integrated into the outer membrane). Most preferably it should take up its native fold within the heterologous bleb context.

An efficient strategy to modulate the composition of a Bleb preparation in this way is to deliver one or more copies of a DNA segment containing an expression cassette comprising a gene encoding said Chlamydia outer membrane protein into the genome of a Gram-negative bacterium.

A non exhaustive list of preferred bacterial species that could be used as a recipient for such a cassette includes: *Bordetella pertussis*, *Borrelia burgdorferi*, *Brucella melitensis*, *Brucella ovis*, *Chlamydia psittaci*, *Chlamydia trachomatis*, *Esherichia coli*, *Haemophilus influenzae*, *Legionella pneumophila*, *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *Pseudomonas aeruginosa* and *Yersinia enterocolitica*. *Neisseria meningitidis*, *Neisseiria gonorrhoeae*, *Moraxella catarrhalis*, *Haemophilus influenzae*, *Pseudomonas aeruginosa*, *Chlamydia trachomatis*, *Chlamydia pneumoniae* are more preferred for this purpose, and

Neisseria gonorrhoeae and *Neisseria meningitidis* are most preferred for making the blebs of this invention. Preferably the *Chlamydia* OMPs are expressed heterologously, and in such situations *Chlamydia* strains should not be used to make the blebs of the invention.

The gene(s) contained in the expression cassette may be homologous (or endogenous) (i.e. exist naturally in the genome of the manipulated bacterium) or, preferably, heterologous (i.e. do not exist naturally in the genome of the manipulated bacterium). The introduced expression cassette may consist of unmodified, "natural" promoter/gene/operon sequences or engineered expression cassettes in which the promoter region and/or the coding region or both have been altered. A non-exhaustive list of preferred promoters (preferably strong) that could be used for expression includes the promoters *porA*, *porB*, *lbpB*, *tbpB*, *p110*, *lst*, *hpuAB* from *N. meningitidis* or *N. gonorroheae*, the promoters p2, p5, p4, ompF, p1, ompH, p6, hin47 from *H. influenzae*, the promoters ompH, ompG, ompCD, ompE, ompB1, ompB2, ompA of *M. catarrhalis*, the promoter λpL, *lac*, *tac*, *araB* of *Escherichia coli* or promoters recognized specifically by bacteriophage RNA polymerase such as the *E. coli* bacteriophage T7.

In a preferred embodiment of the invention the expression cassette is delivered and integrated in the bacterial chromosome by means of homologous and/or site specific recombination (as discussed in WO 01/09350 incorporated by reference herein). Integrative vectors used to deliver such genes and/or operons can be conditionally replicative or suicide plasmids, bacteriophages, transposons or linear DNA fragments obtained by restriction hydrolysis or PCR amplification. Integration is preferably targeted to chromosomal regions dispensable for growth *in vitro*. A non exhaustive list of preferred loci that can be used to target DNA integration includes the *porA*, *porB*, *opa*, *opc*, *rmp*, *omp26*, *lecA*, *cps*, *lgtB* genes of *Neisseiria meningitidis* and *Neisseria gonorrhoeae*, the *P1*, *P5*, *hmw1/2*, *IgA-protease*, *fimE* genes of NTHi; the *lecA1*, *lecA2*, *omp106*, *uspA1*, *uspA2* genes of *Moraxella catarrhalis*. Alternatively, the expression cassette used to modulate the expression of bleb component(s) can be delivered into a bacterium of choice by means of episomal vectors such as circular/linear replicative plasmids, cosmids, phasmids, lysogenic bacteriophages or bacterial artificial chromosomes. Selection of the recombination event can be selected by means of selectable genetic marker such as genes conferring

resistance to antibiotics (for instance kanamycin, erythromycin, chloramphenicol, or gentamycin), genes conferring resistance to heavy metals and/or toxic compounds or genes complementing auxotrophic mutations (for instance *pur, leu, met, aro*). Blebs may be made from the resulting modified strain.

The expression of some heterologous proteins in bacterial blebs may require the addition of outer-membrane targeting signal(s). The preferred method to solve this problem is by creating a genetic fusion between a heterologous gene and a gene coding for a resident OMP as a specific approach to target recombinant proteins to blebs. Most preferably, the heterologous gene is fused to the signal peptides sequences of such an OMP.

A particularly preferred application of this invention is the introduction of Chlamydia (trachomatis or pneumoniae) protective antigens (preferably outer membrane proteins) into Gram-negative bacterial blebs (preferably not from Chlamydia strains). This has several advantages including the fact that such blebs (and vaccines comprising them) are extremely suitable for mucosal administration, which is beneficial as a mucosal (IgA) immune response against the Chlamydia antigens present in the bleb will be more protective against Chlamydia infections which manifest themselves in the mucosa. Recombinant bacteria capable of producing blebs of the invention, processes of making such bacteria, and processes of making bleb preparations are further aspects of this invention.

Chlamydia trachomatis antigens integrated into a Gram negative bacterial bleb

A particularly preferred embodiment is in the field of the prophylaxis or treatment of sexually-transmitted diseaseses (STDs). It is often difficult for practitioners to determine whether the principal cause of a STD is due to gonococcus or *Chlamydia trachomatis* infection. These two organisms are major causes of salpingitis – a disease which can lead to sterility in the host. It would be useful if a STD could be vaccinated against or treated with a combined vaccine effective against disease caused by both organisms. The Major Outer Membrane Protein (MOMP or OMP1 or OMPI) of *C. trachomatis* has been shown to be the target of protective antibodies. However, the structural integrity of this integral membrane protein is important for inducing such antibodies. In addition, the epitopes recognised by these

antibodies are variable and define more than 10 serovars. The bleb context of the invention allows the proper folding of one or more MOMP or other Chlamydia membrane proteins for vaccine purposes. The engineering of (preferably) a gonococcal strain expressing one or more *C. trachomatis* MOMP serovars and/or one or more other protective Chlamydia OMPs in the outer membrane, and the production of blebs therefrom, produces a single solution to the multiple problems of correctly folded membrane proteins, the presentation of sufficient MOMP serovars and/or other Chlamydia OMPs to protect against a wide spectrum of serovars, and the simultaneous prophylaxis/treatment of gonococcal infection (and consequently the non-requirement of practitioners to initially decide which organism is causing particular clinical symptoms – both organisms can be vaccinated against simultaneously thus allowing the treatment of the STD at a very early stage). Preferred loci for gene insertion in the gonoccocal chromosome are give above. Other preferred, protective *C. trachomatis* genes that could be incorporated are HMWP, PmpG and those OMPs disclosed in WO 99/28475 (incorporated by reference herein).

A particularly preferrred embodiment of the invention provides a Gram-negative bacterial bleb (preferably gonococcal) presenting on its surface the PorB outer membrane protein (see below) from *Chlamydia trachomatis*. A bacterial strain capable of producing such a bleb is a further aspect of the invention.

PorB *Chlamydia trachomatis* serovar D (D/UW-3/Cx) DNA sequence

ATGAGTAGCAAGCTAGTGAACTATCTCCGTTTGACTTTCCTATCTTTTTTAGGGATCGCATCTACTTCATTAGACGCTAT
GCCTGCGGGGAATCCGGCGTTTCCAGTCATCCCGGGGATTAATATTGAACAGAAAAATGCCTGTTCTTTCGATTTATGTA
ATTCTTATGATGTACTATCCGCACTGTCCGGTAACCTGAAGCTCTGCTTCTGCGGAGATTATATCTTTTCAGAAGAAGCT
CAGGTAAAAGATGTCCCTGTCGTTACCTCTGTGACAACAGCTGGGGTTGGTCCTTCTCCTGATATTACTTCGACAACCAA
AACGCGAAATTTCGATCTCGTGAACTGTAATCTCAATACAAACTGTGTAGCTGTAGCTTTTTCCCTTCCTGATCGTTCGC
TGAGCGCGATTCCTCTGTTTGATGTGAGTTTCGAAGTGAAAGTAGGAGGACTGAAACAATACTACCGCCTTCCCATGAAT
GCCTATCGAGACTTCACCTCGGAACCTCTCAATTCTGAATCAGAAGTTACGGACGGGATGATTGAAGTACAGTCCAATTA
CGGATTTGTTTGGGATGTTAGCTTGAAAAAAGTCATATGGAAAGATGGCGTTTCCTTTGTAGGCGTCGGTGCAGACTATC
GCCATGCTTCTTGCCCTATTGACTACATCATTGCAAACAGTCAAGCTAATCCAGAAGTATTCATCGCTGACTCGGATGGG
AAACTGAACTTCAAGGAGTGGAGTGTCTGCGTAGGTCTTACTACCTATGTGAATGACTACGTTCTTCCTTACTTAGCGTT
TTCTATAGGGAGTGTTTCTCGCCAAGCTCCGGACGACAGCTTCAAAAAATTAGAAGATCGCTTCACTAACCTCAAATTTA
AAGTTCGTAAAATTACCAGCTCTCATCGTGGAAACATCTGCATCGGAGCGACAAACTATGTCGCCGATAACTTCTTCTAC
AACGTAGAAGGAAGATGGGGAAGCCAGCGCGCTGTGAACGTCTCCGGAGGATTCCAATTCTAA

Translated amino acid sequence

MSSKLVNYLR LTFLSFLGIA STSLDAMPAG NPAFPVIPGI
NIEQKNACSF DLCNSYDVLS ALSGNLKLCF CGDYIFSEEA QVKDVPVVTS
VTTAGVGPSP DITSTTKTRN FDLVNCNLNT NCVAVAFSLP DRSLSAIPLF
DVSFEVKVGG LKQYYRLPMN AYRDFTSEPL NSESEVTDGM IEVQSNYGFV
WDVSLKKVIW KDGVSFVGVG ADYRHASCPI DYIIANSQAN PEVFIADSDG
KLNFKEWSVC VGLTTYVNDY VLPYLAFSIG SVSRQAPDDS FKKLEDRFTN

LKFKVRKITS SHRGNICIGA TNYVADNFFY NVEGRWGSQR AVNVSGGFQF

The presence of PorB in the blebs means that the antigen can be mucosally administered more easily, and provides more effective protection than if administered alone.

The present invention additionally provides a Gram-negative bacterial bleb (preferably gonococcal) presenting on its surface one or more of the following proteins from *Chlamydia trachomatis*, or *C. trachomatis* PorB in combination with one or more of the following proteins. It will be clear to a skilled person that instead of the sequences below (and the PorB sequence above), the natural analogue of the sequences from other *C. trachomatis* serovars or serotypes could be used, as could genes encoding functional analogues of the proteins comprising insertions, deletions or substitutions from the recited sequences which unaffect the immunological properties of the encoded protein. Preferably a sequence from a serovar D strain should be selected. A bacterial strain capable of producing such a bleb is a further aspect of the invention.

>gi|6578118|gb|AAC68456.2| predicted Protease containing IRBP and DHR domains [Chlamydia trachomatis]
MKMNRIWLLLLTFSSAIHSPVQGESLVCKNALQDLSFLEHLLQVKYAPKTWKEQYLGWDLVQSSVSAQQK
LRTQENPSTSFCQQVLADFIGGLNDFHAGVTFFAIESAYLPYTVQKSSDGRFYFVDIMTFSSEIRVGDEL
LEVDGAPVQDVLATLYGSNHKGTAAEESAALRTLFSRMASLGHKVPSGRTTLKIRRPFGTTREVRVKWRY
VPEGVGDLATIAPSIRAPQLQKSMRSFFPKKDDAFHRSSSLFYSPMVPHFWAELRNHYATSGLKSGYNIG
STDGFLPVIGPVIWESEGLFRAYISSVTDGDGKSHKVGFLRIPTYSWQDMEDFDPSGPPPWEEFAKIIQV
FSSNTEALIIDQTNNPGGSVLYLYALLSMLTDRPLELPKHRMILTQDEVVDALDWLTLLENVDTNVESRL
ALGDNMEGYTVDLQVAEYLKSFGRQVLNCWSKGDIELSTPIPLFGFEKIHPHPRVQYSKPICVLINEQDF
SCADFFPVVLKDNDRALIVGTRTAGAGGFVFNVQFPNRTGIKTCSLTGSLAVREHGAFIENIGVEPHIDL
PFTANDIRYKGYSEYLDKVKKLVCQLINNDGTIILAEDGSF
>gi|3329331|gb|AE001359.1:101-1906,
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AAAGCTTGGTTTGCAAGAATGCTCTTCAAGATTTGAGTTTTTTAGAGCATTTATTACAGGTTAAATATGC
TCCTAAAACATGGAAAGAGCAATACTTAGGATGGGATCTTGTTCAAAGCTCCGTTTCTGCACAGCAGAAG
CTTCGTACACAAGAAAATCCATCAACAAGTTTTTGCCAGCAGGTCCTTGCTGATTTTATCGGAGGATTAA
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TGAGAAGCTTTTTCCCTAAGAAAGATGATGCGTTTCATCGGTCTAGTTCGCTATTCTACTCTCCAATGGT
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TTTCTTCGGTGACTGATGGGGATGGTAAGAGCCATAAAGTAGGATTTCTAAGAATTCCTACATATAGTTG
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ATGCACTGCTTTCCATGTTGACAGACCGTCCTTTAGAACTTCCTAAACATAGAATGATTCTGACTCAGGA
TGAAGTGGTTGATGCTTTAGATTGGTTAACCCTGTTGGAAAACGTAGACACAAACGTGGAGTCTCGCCTT

GCTCTGGGAGACAACATGGAAGGATATACTGTGGATCTACAGGTTGCCGAGTATTTAAAAAGCTTTGGAC
GTCAAGTATTGAATTGTTGGAGTAAAGGGGATATCGAGTTATCAACGCCTATTCCTCTTTTTGGTTTTGA
GAAGATTCATCCACATCCTCGAGTTCAATACTCTAAACCGATTTGTGTTTTGATCAATGAGCAAGACTTT
TCTTGTGCTGACTTCTTCCCTGTAGTTTTGAAAGACAATGATCGAGCTCTTATTGTTGGTACTCGAACAG
CTGGAGCTGGAGGATTTGTCTTTAATGTGCAGTTCCCAAATAGAACTGGAATAAAAACTTGTTCTTTAAC
AGGATCATTAGCTGTTAGAGAGCATGGTGCCTTCATTGAGAACATCGGAGTCGAACCGCATATCGATCTG
CCTTTTACAGCGAATGATATTCGCTATAAAGGCTATTCCGAGTATCTTGATAAGGTCAAAAAATTGGTTT
GTCAGCTGATCAATAACGACGGTACCATTATTCTTGCGGAAGATGGTAGTTTTTAA

>gi|6578109|gb|AAC68227.2| CHLPN 76kDa Homolog [Chlamydia trachomatis]
MKKYFYKGFVGALLLACGSTNLAFAQASSMDSQLWSVEDLDSYLSSKGFVETRKRDGVLRLAGDVRARWI
YAKEDLETTQTPAKPMLPTNRYRSEFNLYVDYTAANSWMTSKMNWVTIAGGESSAAGLDINRAFLGYRFY
KNPETQAEVFAEIGRSGLGDIFDSDVQFNSNFDGIHLYAARRISEKLPFTMIVHGGPFVVNMAEKEYAWV
VEAILNKLPGNFVVKTSVVDWNTLTAKTNDPADASAAQPAKPNTKYDYLVWQWLVGKSTAMPWFNGQTKN
LYTYGAYLFNPLAEIPENWKQSTTPTTKITNGKENHAWFIGCSLGGVRRAGDWSATVRYEYVEALAIPEI
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TATGCAAAAGAGGATCTTGAGACAACTCAGACTCCTGCTAAACCTATGTTACCTACCAATCGGTATCGTA
GTGAATTCAATTTGTATGTGGATTACACCGCTGCTAATAGTTGGATGACTTCGAAAATGAATTGGGTAAC
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TCCTTTCACCATGATTGTTCATGGTGGTCCTTTTGTCGTGAATATGGCAGAGAAAGAGTATGCTTGGGTC
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TAACAGCAAAAACGAATGATCCAGCAGACGCAAGCGCTGCACAACCAGCTAAACCTAATACCAAGTACGA
TTATTTAGTATGGCAATGGTTGGTTGGGAAGAGCACAGCTATGCCATGGTTTAATGGACAAACAAAAAAT
CTTTACACTTACGGAGCCTATCTCTTTAATCCATTAGCGGAAATACCAGAGAACTGGAAACAATCAACAA
CTCCTACAACCAAAATTACAAATGGTAAGGAAAACCATGCTTGGTTCATCGGCTGCTCTCTAGGCGGTGT
TCGACGAGCTGGAGACTGGTCTGCAACAGTTCGTTATGAGTATGTTGAAGCTTTAGCGATTCCAGAAATT
GATGTCGCGGGTATTGGTCGCGGAAACCAAATGAAATATTGGTTTGCTCAAGCTATCAAACAAGGATTGG
ATCCTAAAGAATCTAACGGCTTTACTAACTATAAAGGAGTTTCCTATCAGTTTGTTATGGGTCTGACAGA
TTCGGTTTCTTTCCGAGCTTATGCTGCTTATTCTAAGCCTGCTAACGATAACCTTGGTAGCGACTTCACC
TATCGTAAGTATGACCTAGGTTTAATTTCTTCATTCTAA

>gi|3329350|gb|AAC68472.1| Putative Outer Membrane Protein I [Chlamydia trachomatis]
MRPDHMNFCCLCAAILSSTAVLFGQDPLGETALLTKNPNHVVCTFFEDCTMESLFPALCAHASQDDPLYV
LGNSYCWFVSKLHITDPKEALFKEKGDLSIQNFRFLSFTDCSSKESSPSIIHQKNGQLSLRNNGSMSFCR
NHAEGSGGAISADAFSLQHNYLFTAFEENSSKGNGGAIQAQTFSLSRNVSPISFARNRADLNGGAICCSN
LICSGNVNPLFFTGNSATNGGAICCISDLNTSEKGSLSLACNQETLFASNSAKEKGGAIYAKHMVLRYNG
PVSFINNSAKIGGAIAIQSGGSLSILAGEGSVLFQNNSQRTSDQGLVRNAIYLEKDAILSSLEARNGDIL
FFDPIVQESSSKESPLPSSLQASVTSPTPATASPLVIQTSANRSVIFSSERLSEEEKTPDNLTSQLQQPI
ELKSGRLVLKDRAVLSAPSLSQDPQALLIMEAGTSLKTSSDLKLATLSIPLHSLDTEKSVTIHAPNLSIQ
KIFLSNSGDENFYENVELLSKEQNNIPLLTLSKEQSHLHLPDGNLSSHFGYQGDWTFSWKDSDEGHSLIA
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>gi|3329279|gb|AAC68408.1| Putative Outer Membrane Protein D [Chlamydia trachomatis]
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>gi|3329169|gb|AAC68308.1| Outer Membrane Protein Analog [Chlamydia trachomatis]

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>gi|3328866|gb|AAC68034.1| Sulfite Reductase [Chlamydia trachomatis]
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>gi|3328843|gb|AAC68011.1| Putative outer membrane protein C [Chlamydia trachomatis]
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>gi|3328815|gb|AAC67986.1| hypothetical protein [Chlamydia trachomatis]
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>gi|3328587|gb|AAC67774.1| CMP-2-keto-3-deoxyoctulosonic acid synthetase [Chlamydia trachomatis]
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>gi|3329039|gb|AAC68197.1| Thio:disulfide Interchange Protein [Chlamydia trachomatis]
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>gi|3329000|gb|AAC68161.1| Yop proteins translocation lipoprotein J [Chlamydia trachomatis]
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>gi|3328905|gb|AAC68071.1| hypothetical protein [Chlamydia trachomatis]
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>gi|3328855|gb|AAC68022.1| hypothetical protein [Chlamydia trachomatis]
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>gi|3329347|gb|AAC68470.1| Putative Outer Membrane Protein H [Chlamydia trachomatis]
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>gi|3328874|gb|AAC68042.1| 60kDa Cysteine-Rich OMP [Chlamydia trachomatis]
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>gi|3328841|gb|AAC68010.1| Putative outer membrane protein B [Chlamydia trachomatis]
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>gi|3328840|gb|AAC68009.1| Putative outer membrane protein A [Chlamydia trachomatis]
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FQGAWHFSWQPNTINNTKQKILRASWLPTGEYVLESNRVGRAVPNSLWSTFLLLQTASHNLGDHLCNNRS
LIPTSYFGVLIGGTGAEMSTHSSEEESFISRLGATGTSIIRLTPSLTLSGGGSHMFGDSFVADLPEHITS
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>gi|3328763|gb|AAC67938.1| O-Sialoglycoprotein Endopeptidase family [Chlamydia trachomatis]
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AFAQGLSLAKNVPLVGYSSLEGYLSLGQEEEALLLPLGKKGGIVALNSELSLDGFLLTDTTSTPGILLSY
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>gi|6578102|gb|AAC67897.2| ATP Synthase Subunit K [Chlamydia trachomatis]
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>gi|3328718|gb|AE001303.1:c956-531,
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TTCTAA

>gi|3329252|gb|AAC68382.1| S14 Ribosomal Protein [Chlamydia trachomatis]
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>gi|3522908|gb|AE001351.1:2436-2741,
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TCTCAATAAAATGAGAAGAGATACTTCTCCTTCTCGTTTACATAATAGATGCCTATTAACAGGCCGTCCT
CGTGGATACCTTAGAAAGTTTGCTATCTCAAGAATTTGTTTTAGACAAATGGCTTCTATGGGAGATATCC

CAGGCGTTGTGAAAGCAAGTTGGTAG

>gi|3329133|gb|AAC68276.1| Major Outer Membrane Protein [Chlamydia trachomatis]
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DRVLKTDVNKEFQMGAKPTTDTGNSAAPSTLTARENPAYGRHMQDAEMFTNAACMALNIWDRFDVFCTLG
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ASFQYAQSKPKVEELNVLCNAAEFTINKPKGYVGKEFPLDLTAGTDAATGTKDASIDYHEWQASLALSYR
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>gi|3328987|gb|AAC68150.1| hypothetical protein [Chlamydia trachomatis]
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>gi|3328972|gb|AAC68136.1| Apolipoprotein N-Acetyltransferase [Chlamydia trachomatis]
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NQPVLESLLPNKSWGEFFTNLDWIQAIAERYQCTVIMGMERWENKGGILHLYNAAECVSREGEITSYDKR
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ADILVNLTNDGWYPRSRLPLVHFYHGMLRNQELGIPCIRACRTGVSAAVDSLGRIVGILPWESRTCPVST
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>gi|3328612|gb|AAC67797.1| Fructose-6-P Phosphotransferase [Chlamydia trachomatis]
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SPLKVGVMLSGGPAPGGHNVILGLLHSIKKLHPNSQLLGFIRNGEGLLNNNTVEITDEFIEEFRNSGGFN
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HLFLDLTFGFDTATKFYSSIISNISRDALSCKGHYHFIKLMGRSSSHITLECALQTHPNIALIGEEIAEK
SISLETLIHDICETIADRAAMGKYHGVILIPEGVIEFIPEIQSLVKEIESIPEQENLYQALSLSSQQLLC
QFPEDICHQLLYNRDAHGNVYVSKISVDKLLIHLVRQHLETHFRQVPFNAISHFLGYEGRSGTPTHFDNV
YSYNLGYGAGVLVFNRCNGYLSTIEGLTSPIEKWRLRALPIVRMLTTKQGKDSKHYPLIKKRLVDIASPV
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>gi|3328517|gb|AAC67709.1| hypothetical protein [Chlamydia trachomatis]
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>gi|3328482|gb|AAC67677.1| L28 Ribosomal Protein [Chlamydia trachomatis]
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LVDKLGLDQVVARAKSKGF
>gi|3328480|gb|AE001283.1:c2251-1982,
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>gi|3328436|gb|AAC67635.1| SS DNA Binding Protein [Chlamydia trachomatis]
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>gi|3328434|gb|AE001279.1:1060-1533,
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>gi|3328411|gb|AAC67611.1| hypothetical protein [Chlamydia trachomatis]
MKKQEKMHPQNLLKVFIFFLAFFISYPSAEAHSPLQSSIQEKILTARPGDYAVLSRGSQKFFFLIRQSSS
EATWVEMSEFASLTQQEKKLVEQSSWKNAFHQLQSSKKVYLLRISKNPLMIFVLKNAQWMPLSEKDPLPF
FVKILRLPLSPAPSHLIKYKGKERTPWSPRTSLNGELITLPSSAWISVWPKDSSPLSEKNILIYFSNNER
LAFPLWTSIDTPTGTVIIKTIEMGHQAASSYPALPNF
>gi|3522886|gb|AE001277.1:c6191-5448,
ATGAAAAAGCAAGAAAAAATGCACCCTCAAAACCTTCTTAAAGTTTTTATTTTTTTCTTGGCATTTTTCA
TATCCTATCCCTCGGCTGAAGCCCATTCTCCTCTCCAATCATCAATCCAAGAAAAAATTCTAACTGCCCG
CCCCGGAGACTATGCCGTCTTAAGCCGAGGATCTCAAAAATTTTTCTTTTTAATTCGCCAAAGTTCTTCG
GAAGCGACTTGGGTCGAAATGTCTGAATTTGCCTCCCTAACACAGCAAGAAAAAAAAATTAGTAGAACAGT
CTTCCTGGAAGAATGCCTTCCATCAACTCCAATCTTCAAAAAAAGTGTACTTGTTACGAATTTCCAAAAA
TCCTCTTATGATTTTTGTTCTCAAAAATGCGCAATGGATGCCTCTCTCAGAAAAAGATCCTTTGCCTTTC
TTTGTAAAAATCCTTCGACTCCCTTTATCTCCAGCCCCCTCTCACTTAATTAAATACAAAGGGAAAGAAC
GCACCCCCTGGTCTCCGCGAACATCTTTGAATGGAGAACTCATAACCCTTCCTTCCAGTGCTTGGATTTC
TGTTTGGCCAAAAGATTCTTCTCCTCTATCAGAAAAAAATATTCTCATATATTTTTCTAACAATGAACGT
TTAGCGTTTCCTCTATGGACTAGTATTGATACTCCTACAGGGACAGTGATTATTAAGACTATTGAAATGG
GGCACCAAGCCGCCTCCTCCTATCCAGCTCTTCCCAATTTCTAG

crpA, CHLTR 15 kD cysteine-rich protein (*Chlamydia trachomatis* serovar D (D/UW-3/Cx)

DNA sequence
AATATGAGCACTGTACCCGTTGTTCAAGGAGCTGGATCTTCCAATTCGGCACAGGATATTTCCACTAGA
CCATTAACACTGAAAGAGCGTATATCGAATCTTCTATCTTCCACTGCATTTAAGGTGGGATTAGTGGTG
ATAGGACTACTTTTAGTGATTGCTACTTTGATATTCCTAGTTTCGGCAGCTTCGTTTGTAAATGCCATC
TATCTAGTAGCTATTCCTGCTATTTTGGGATGCGTGAATATCTGCGTAGGAATTTTATCCATGGAAGGA
CACTGTTCTCCGGAGAGATGGATCTTATGTAAGAAGGTATTAAAGACTTCAGAAGATATCATCGATGAT
GGGCAGATAAACAACTCTAATAAAGTGTTTACTGATGAGAGGTTGAATGCCATAGGTGGGGTAGTGGAA
TCTCTATCTAGAAGAAATAGTCTGGTGGATCAGACCCAATGA
Translated amino acid sequence
NMS TVPVVQGAGS SNSAQDISTR PLTLKERISN LLSSTAFKVG
LVVIGLLLVI ATLIFLVSAA SFVNAIYLVA IPAILGCVNI CVGILSMEGH
CSPERWILCK KVLKTSEDII DDGQINNSNK VFTDERLNAI GGVVESLSRR
NSLVDQTQ*

OmcA, CHLTR 9 kD cystein-rich outer membrane complex lipoprotein (*Chlamydia trachomatis* serovar D (D/UW-3/Cx)
DNA sequence

GGGCTAGTTTCTTTTATTGTTAAAAGAATTGCTTTTATCGATAAAAGAAACTTCAAGAGCCCTTTTCTAGAAAGGAGTCT
GGAAGTTATGAAAAAAACTGCTTTACTCGCTGCTTTATGTAGTGTTGTTTCTTTAAGTAGTTGTTGTCGTATCGTTGACT
GTTGCTTCGAAGATCCATGCGCACCTATCCAATGTTCACCTTGTGAATCTAAGAAGAAAGACGTAGACGGTGGTTGCAAC
TCTTGTAACGGGTATGTCCCAGCTTGCAAACCTTGCGGAGGGGATACGCACCAAGATGCTAAACATGGCCCTCAAGCTAG
AGGAATTCCAGTTGACGGCAAATGCAGACAATAG

Translated amino acid sequence

GL VSFIVKRIAF IDKRNFKSPF
LERSLEVMKK TALLAALCSV VSLSSCCRIV DCCFEDPCAP IQCSPCESKK
KDVDGGCNSC NGYVPACKPC GGDTHQDAKH GPQARGIPVD GKCRQ*

cutE, apolipoprotein N-acyltransferase (*Chlamydia trachomatis* serovar D (D/UW-3/Cx)

DNA sequence

GCTAGTAAGGGAGCCCCTTTAGTGTTTAAACTTGTGTCATACATCATCCTTTCTTGGGTGCTGGTCTGTTTGGCTCAGCC
GGATGTAAGTGTTGTAGCTTCTGTTGTTAGTTGTATTTGCGGTTACAGCTTACTTTGGGCTGGGCTTTTTGCTTTAGTAG
AGCAATTATCTTGGAAGAAAGTTTGGTGCATCGCTTTTATTTGGACTTGGACTGTCGAAGGCGCTCATTTCTCTTGGATG
CTTGAAGATCTTTATGTAGGGACAAGCATCTATTTTGTTTGGGGTATACTGCTTTCTTATCTCGCCACCCTATTTGCTAG
TTTTTCTTGTTTGGTTGTGTGGTGTTGTCGCAAGCAATATAGGGGAGCTCTTGTTTGGCTTCCAGGGGTTTGGGTGGCGA
TAGAAGCAATACGCTATTATGGGTTGCTTTCAGGAGTTTCTTTTGATTTTATTGGCTGGCCTCTTACAGCGACAGCCTAT
GGCCGGCAATTCGGCAGCTTTTTTGGATGGGCTGGACAAAGCTTTCTAGTTATTGCTGCCAATATATGCTGTTTTGCAGT
ATGTTTATTAAAACACTCTTTTTCCAAAGGTTTGTGGTTGACGTTGTGCGCGTTCCCTTATCTGTTAGGCGGAGCGCATT
ACGAATACCTAAAGAAGCATTTTTCCGACTCTGAAGTGCTTCGAGTTGCCATCGTGCAGCCTGGATATAGTCCTCATATG
CATGCAGGGAGGACGGCTAGTGCTATTTGGAGAGGTTTGGTTTCTTTGTGCCAGACTATTCAAACTCCTGTAGATGTGAT
CGTTTTCCCAGAAGTAAGTGTTCCTTTTGGCTTACATAGACAAGCCTATACTCTTCATGAAAATCAGCCTGTATTAGAAA
GTTTGCTTCCTAACAAATCTTGGGGCGAGTTTTTCACAAATTTGGATTGGATCCAAGCGATAGCTGAACGTTATCAATGC
ACCGTTATCATGGGAATGGAACGATGGGAAAATAAAGGGGGAATACTGCATTTGTATAATGCTGCTGAATGCGTATCGCG
AGAAGGGGAAATAACTAGCTATGATAAGCGGATTCTTGTTCCTGGAGGTGAGTACATCCCTGGAGGGAAAATAGGTTTTT
CCTTGTGTCAAACCTTTTTCCCAGAATTTGCTCTTCCCTTTCAACGTTTGCCAGGAGAGTTTTCTGGAGTTGTGAATATA
ACAGAGCGAATAAAAGCTGGGATCTCTATTTGTTATGAGGAGACATTTGGGTATGCAATTCGCCCTTACAAAAGGCAACA
AGCCGATATTTTAGTAAATCTTACTAATGACGGTTGGTATCCGCGTTCAAGGCTGCCTCTAGTACATTTTTATCATGGCA
TGTTACGTAATCAAGAGTTGGGTATACCTTGTATTCGCGCCTGTCGCACAGGAGTTTCTGCTGCAGTGGATTCTTTGGGT
AGAATTGTCGGCATACTTCCCTGGGAATCGAGAACTTGCCCAGTTTCTACAGGAGTACTCCAAGTTTCCGTCCCTCTTTA
CAGTTATCATACTGTATATGCAAGGCTGGGTGATGCTCCTCTGTTACTGATTGCAGTTTGTTCGGTTATCGGAGCGATTG
CCTATTTTTATAGGAAAAAGAAAGAGACCCCACCACAAACATTTTTTTGA

Translated amino acid sequence

ASKG
APLVFKLVSY IILSWVLVCL AQPDVSVVAS VVSCICGYSL LWAGLFALVE
QLSWKKVWCI AFIWTWTVEG AHFSWMLEDL YVGTSIYFVW GILLSYLATL
FASFSCLVVW CCRKQYRGAL VWLPGVWVAI EAIRYYGLLS GVSFDFIGWP
LTATAYGRQF GSFFGWAGQS FLVIAANICC FAVCLLKHSF SKGLWLTLCA
FPYLLGGAHY EYLKKHFSDS EVLRVAIVQP GYSPHMHAGR TASAIWRGLV
SLCQTIQTPV DVIVFPEVSV PFGLHRQAYT LHENQPVLES LLPNKSWGEF
FTNLDWIQAI AERYQCTVIM GMERWENKGG ILHLYNAAEC VSREGEITSY
DKRILVPGGE YIPGGKIGFS LCQTFFPEFA LPFQRLPGEF SGVVNITERI
KAGISICYEE TFGYAIRPYK RQQADILVNL TNDGWYPRSR LPLVHFYHGM
LRNQELGIPC IRACRTGVSA AVDSLGRIVG ILPWESRTCP VSTGVLQVSV
PLYSYHTVYA RLGDAPLLLI AVCSVIGAIA YFYRKKKETP PQTFF*

pal, peptidoglycan-associated lipoprotein (*Chlamydia trachomatis* serovar D (D/UW-3/Cx)

DNA sequence

G AAAATTGTTA TAGGATCAGG
AGAGAAACGT TTCCCATGCT GGGGAGCATT TCCTTCACAA CATATAAAGA
AAACCTCATG AGAAAGACTA TTTTTAAAGC GTTTAATTTA TTATTCTCCC
TTCTTTTTCT TTCTTCATGC TCTTATCCTT GCAGAGATTG GGAATGCCAT
GGTTGCGACT CCGCAAGACC TCGTAAATCC TCTTTTGGAT TCGTACCTTT
CTACTCCGAT GAAGAAATTC AACAAGCTTT TGTTGAAGAT TTTGATTCCA
AAGAAGAGCA GCTGTACAAA ACGAGCGCAC AGAGTACCTC TTTCCGAAAT
ATCACTTTCG CTACAGATAG TTATTCTATT AAAGGAGAGG ATAACCTCAC
GATTCTTGCA AGCTTAGTTC GTCATTTGCA TAAATCTCCT AAAGCTACGC

TATATATAGA GGGCCATACA GATGAACGTG GAGCTGCAGC TTATAACCTA
GCTTTAGGAG CTCGTCGTGC GAATGCTGTA AAACAATACC TCATCAAACA
GGGAATCGCT GCAGACCGCT TATTCACTAT TTCTTACGGA AAAGAACATC
CTGTTCATCC AGGCCATAAT GAATTAGCTT GGCAACAAAA TCGTCGTACT
GAATTTAAGA TCCATGCTCG CTAA

Translated amino acid sequence

ENCYRIR
RETFPMLGSI SFTTYKENLM RKTIFKAFNL LFSLLFLSSC SYPCRDWECH
GCDSARPRKS SFGFVPFYSD EEIQQAFVED FDSKEEQLYK TSAQSTSFRN
ITFATDSYSI KGEDNLTILA SLVRHLHKSP KATLYIEGHT DERGAAAYNL
ALGARRANAV KQYLIKQGIA ADRLFTISYG KEHPVHPGHN ELAWQQNRRT
EFKIHAR*

The following *Chlamydia trachomatis* outer membrane proteins (full sequences above) are disclosed for the first time as being useful in a *C. trachomatis* vaccine. A vaccine comprising one or more of these proteins (or native or functional analogues thereof) is a further aspect of this invention (particularly in the context of being presented on the surface of a bleb).

Amino acid sequences:

>gi|6578118|gb|AAC68456.2| predicted Protease containing IRBP and DHR domains [Chlamydia trachomatis]
>gi|6578109|gb|AAC68227.2| CHLPN 76kDa Homolog [Chlamydia trachomatis]
>gi|3328866|gb|AAC68034.1| Sulfite Reductase [Chlamydia trachomatis]
>gi|3328815|gb|AAC67986.1| hypothetical protein [Chlamydia trachomatis]
>gi|3328587|gb|AAC67774.1| CMP-2-keto-3-deoxyoctulosonic acid synthetase [Chlamydia trachomatis]
>gi|3329039|gb|AAC68197.1| Thio:disulfide Interchange Protein [Chlamydia trachomatis]
>gi|3329000|gb|AAC68161.1| Yop proteins translocation lipoprotein J [Chlamydia trachomatis]
>gi|3328905|gb|AAC68071.1| hypothetical protein [Chlamydia trachomatis]
>gi|3328884|gb|AAC68051.1| Phosphatidate Cytidylytransferase [Chlamydia trachomatis]
>gi|3328855|gb|AAC68022.1| hypothetical protein [Chlamydia trachomatis]
>gi|3328772|gb|AAC67946.1| hypothetical protein [Chlamydia trachomatis]
>gi|3328763|gb|AAC67938.1| O-Sialoglycoprotein Endopeptidase family [Chlamydia trachomatis]
>gi|6578102|gb|AAC67897.2| ATP Synthase Subunit K [Chlamydia trachomatis]
>gi|3329252|gb|AAC68382.1| S14 Ribosomal Protein [Chlamydia trachomatis]
>gi|3328987|gb|AAC68150.1| hypothetical protein [Chlamydia trachomatis]
>gi|3328972|gb|AAC68136.1| Apolipoprotein N-Acetyltransferase [Chlamydia trachomatis]
>gi|3328612|gb|AAC67797.1| Fructose-6-P Phosphotransferase [Chlamydia trachomatis]
>gi|3328517|gb|AAC67709.1| hypothetical protein [Chlamydia trachomatis]
>gi|3328482|gb|AAC67677.1| L28 Ribosomal Protein [Chlamydia trachomatis]
>gi|3328436|gb|AAC67635.1| SS DNA Binding Protein [Chlamydia trachomatis]
>gi|3328411|gb|AAC67611.1| hypothetical protein [Chlamydia trachomatis]

Again, when such blebs are present in a vaccine formulation they may be more protective against *Chlamydia trachomatis* infection than the use of the protein in isolation.

Particularly beneficial pairs of *Chlamydia trachomatis* antigens are further preferred embodiments of this invention. Thus in a further aspect a Gram-negative bleb (preferably from gonococcus) is provided presenting on its surface both the PorB and PmpG outer membrane proteins from *Chlamydia trachomatis*. Furthermore, a Gram-negative bleb (preferably from gonococcus) is provided presenting on its surface both the PorB and MOMP (from one or more serovars) outer membrane proteins from *Chlamydia trachomatis*. Lastly, a Gram-negative bleb (preferably from gonococcus) is provided presenting on its surface both the PmpG and MOMP (from one or more serovars) outer membrane proteins from *Chlamydia trachomatis*.

By MOMP (or OMP1 or OMP I) from one or more (1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) serovars it is preferred that one or more should be selected from a list serovars consisting of: B, Ba, D, Da, E, L1, L2, L2a, F, G, K, L3, A, C, H, I, Ia, & J; more preferably from a list consisting of D, E, F, G, K, H, I, & J. Most preferably one or more MOMPs should at least comprise MOMP from serovar D or E (most preferably D). A further preferred strategy is the selection of one or more MOMP from each of the following 3 serogroups: B-serogroup (consisting of serovars B, Ba, D, Da, E, L1, L2 and L2a, and preferably selected from serovars D, Da, & E); F-G-serogroup (consisting of serovars F and G); and C-serogroup (consisting of servars A, C, H, I, Ia, J, K, and L3, and preferably selected from serovars H, I, Ia, J and K).

Most preferably the genes for the *Chlamydia trachomatis* antigens should be inserted at the PorA locus of *Neisseria* (preferably gonococcus).

Such a prepartion formulated as a vaccine may give enhanced protection to a host against *Chlamydia trachomatis* than when a single antigen is administered.

Preferably the bleb has been derived from a strain (preferably gonococcus) which has been modified to upregulate one or more protective outer membrane antigens (as described below).

Preferably the bleb has been derived from a strain (preferably gonococcus) which has been modified to downregulate one or more immunodominant variable or non-protective outer membrane antigens (as described below).

Preferably the blebs are derived from a strain (preferably gonococcus) which has a detoxified lipid A portion of bacterial LPS, due to the strain having been engineered to reduce or switch off expression of one or more genes which cause LPS

to be toxic (preferably selected from the following genes, or homologues thereof htrB, msbB and lpxK; see section below).

Preferably the blebs are derived from a strain (preferably gonococcus) which has a detoxified lipid A portion of bacterial LPS, due to the strain having been engineered to express at a higher level of one or more genes producing a gene product that is capable of detoxifying LPS (preferably selected from the following genes, or homologues thereof: pmrA, pmrB, pmrE and pmrF; see section below).

Vaccine compositions comprising the bleb of the invention and a pharmaceutically suitable excipient or carrier is also envisaged. Preferably the vaccine additionally comprises a mucosal adjuvant. Mucosal adjuvants are well known in the art (see Vaccine Design "The subunit and adjuvant approach" (eds Powell M.F. & Newman M.J.) (1995) Plenum Press New York). A preferred mucosal adjuvant is LT2 (or LTII, which can be split into LTIIa and LTIIb – see Martin et al. Infection and Immunity, 2000, 68:281-287). Preferably such vaccines should be formulated and administered as described below in "vaccine formulations".

The content of blebs per dose in the vaccine will typically be in the range 1-100μg, preferably 5-50μg, most typically in the range 5 - 25μg.

Optimal amounts of components for a particular vaccine can be ascertained by standard studies involving observation of appropriate immune responses in subjects. Following an initial vaccination, subjects may receive one or several booster immunisations adequately spaced.

A method of preventing *Chlamydia trachomatis* infection in a host is also provided comprising the steps of administering an effective amount of the above vaccine to a host in need thereof. Preferably the vaccine is mucosally administered via either a intranasal, oral, intradermal or intravaginal route.

Chlamydia pneumoniae antigens integrated into a Gram negative bacterial bleb

In a further aspect, the invention provides a Gram-negative bleb presenting on its surface a protective antigen from *Chlamydia pneumoniae. Neisseria meningitidis*, *Moraxella catharralis*, and *Haemophilus influenzae* are preferred species for the

production of said bleb. A bacterial strain capable of producing such a bleb is a further aspect of the invention. Such protective antigens are preferably one or more of those listed below:

1) Cell Envelope: Membrane Proteins, Lipoproteins and Porins

Gene:	Protein Function:
yaeT	OMP85 homolog
60IM	60 kD inner membrane protein
lgt	prolipoprotein diacylglyceryl transferase
crpA	CHLTR 15 kD cysteine-rich protein
omcB	60 kD cysteine-rich outer membrane complex protein
omcA	9 kD cysteine-rich outer membrane complex lipoprotein
cutE	apolipoprotein N-acetyltransferase
ompA	major outer membrane protein
pal	peptidoglycan-associated lipoprotein
porB	outer membrane protein analog

2) Coding Genes (Not in C. trachomatis)

Gene:	Protein Function:
yqfF	conserved hypothetical inner membrane protein
yxjG	hypothetical protein
guaA	GMP synthase
guaB	inosine 5'-monophosphate dehydrogenase
argR	similarity to arginine repressor
CPn0232	similarity to 5'-methylthioadenosine nucleosidase
CPn0251	conserved hypothetical protein
CPn0278	conserved outer membrane lipoprotein protein/a>
CPn0279	possible ABC transporter permease
yxjG	hypothetical protein
yqeV	hypothetical protein
CPn0486	hypothetical proline permease
CPn0505	3-methyladenine DNA glycosylase
CPn0562	CHLPS 43 kDa protein
CPn0585	similarity to CHLPS IncA
yvyD	conserved hypothetical protein
CPn0608	uridine 5'-monophosphate synthase
CPn0735	uridine kinase
CPn0907	CutA-like periplasmic divalent cation tolerance protein
CPn0927	CHLPS 43 kDa protein
CPn0928	CHLPS 43 kDa protein
CPn0929	CHLPS 43 kDa protein
CPn0980	similar to S. cerevisiae 52.9 kDa protein
bioA	adenosylmethionine-8-amino-7-oxononanoate aminotransferase
bioD	dethiobiotin synthetase
bioB	biotin synthase
CPn1045	conserved hypothetical membrane protein
CPn1046	tryptophan hydroxylase

3) Chlamydia-Specific Proteins

Gene:	Protein Function:

pmp_1	polymorphic outer membrane protein
pmp_2	polymorphic outer membrane protein
pmp_3	polymorphic outer membrane protein
pmp_3	polymorphic outer membrane protein
pmp_4	polymorphic outer membrane protein
pmp_4	polymorphic outer membrane protein
pmp_5	polymorphic outer membrane protein
pmp_5	polymorphic outer membrane protein
CPn0133	CHLPS hypothetical protein
CPn0186	similarity to IncA
incB	inclusion membrane protein B
incC	inclusion membrane protein C
CPn0332	CHLTR T2 protein
ltuB	LtuB protein
pmp_6	polymorphic outer membrane protein
pmp_7	polymorphic outer membrane protein
pmp_8	polymorphic outer membrane protein
pmp_9	polymorphic outer membrane protein
pmp_10	polymorphic outer membrane protein
pmp_10	polymorphic outer membrane protein
pmp_11	polymorphic outer membrane protein
pmp_12	polymorphic outer membrane protein
pmp_13	polymorphic outer membrane protein
pmp_14	polymorphic outer membrane protein
pmp_15	polymorphic outer membrane protein
pmp_16	polymorphic outer membrane protein
pmp_17	polymorphic outer membrane protein
pmp_17	polymorphic outer membrane protein
pmp_17	polymorphic outer membrane protein
pmp_18	polymorphic outer membrane protein
pmp_19	polymorphic outer membrane protein
pmp_20	polymorphic outer membrane protein
euo	CHLPS Euo protein
CPn0562	CHLPS 43 kDa protein homolog
CPn0585	similar to CHLPS inclusion membrane protein A
CPn0728	CHLPN 76 kDa protein homolog
CPn0729	CHLPN 76 kDa protein homolog
gp6D	CHLTR plasmid protein
CPn0927	CHLPS 43 kDa protein homolog
CPn0928	CHLPS 43 kDa protein homolog
CPn0929	CHLPS 43 kDa protein homolog
pmp_21	polymorphic outer membrane protein
ltuA	LtuA protein

(Full sequence information has been published at the Chlamydia Genome Project web site: http://chlamydia-www.berkeley.edu:4231/index.html).

Additional Chlamydia genes, and encoded proteins, suitable for expression in a Gram-negative bacteria for OMV vaccine preparation:

Chlamydia pneumoniae 98kD putative outer membrane protein gene. **WO200026237-A2**

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MURDIN AD

Chlamydia POMP91B precursor gene. **WO200026239-A2**

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Chlamydia antigen CPN100634 full length coding sequence.	**WO200032794 -A2**
Patent Inventors *OOMEN RP* *WANG J* *MURDIN AD*	
Chlamydia antigen CPN100634 gene open reading frame.	**WO200032794 -A2**
Patent Inventors *OOMEN RP* *WANG J* *MURDIN AD*	
Chlamydia antigen CPN100635 full length coding sequence.	**WO200032794 -A2**
Patent Inventors *OOMEN RP* *WANG J* *MURDIN AD*	
Chlamydia antigen CPN100635 gene open reading frame.	**WO200032794 -A2**
Patent Inventors *OOMEN RP* *WANG J* *MURDIN AD*	
Chlamydia antigen CPN100638 full length coding sequence.	**WO200032794 -A2**
Patent Inventors *OOMEN RP* *WANG J* *MURDIN AD*	
Chlamydia antigen CPN100638 gene open reading frame.	**WO200032794 -A2**
Patent Inventors *OOMEN RP* *WANG J* *MURDIN AD*	
Chlamydia antigen CPN100639 full length coding sequence.	**WO200032794 -A2**
Patent Inventors *OOMEN RP* *WANG J* *MURDIN AD*	
Chlamydia antigen CPN100639 gene open reading frame.	**WO200032794 -A2**
Patent Inventors *OOMEN RP* *WANG J* *MURDIN AD*	
Chlamydia antigen CPN100708 full length coding sequence.	**WO200032794 -A2**
Patent Inventors *OOMEN RP* *WANG J* *MURDIN AD*	
Chlamydia antigen CPN100708 gene open reading frame.	**WO200032794 -A2**
Patent Inventors *OOMEN RP* *WANG J* *MURDIN AD*	
C. pneumoniae ATP/ADP translocase coding sequence.	**WO200039157 -A1**
Patent Inventors *DUNN P* *OOMEN RP* *WANG J* *MURDIN AD*	
Chlamydia pneumoniae 98 kDa outer membrane protein CPN100640 gene.	**WO200032784 -A1**
Patent Inventors *DUNN P*	

OOMEN RP *WANG J* *MURDIN AD*	
Chlamydia pneumoniae 98 kDa outer membrane protein coding region.	**WO200032784 -A1**
Patent Inventors *DUNN P* *OOMEN RP* *WANG J* *MURDIN AD*	
DNA encoding a 9 kDa cysteine-rich membrane protein.	**WO200053764 -A1**
Patent Inventors *DUNN P* *OOMEN RP* *WANG J* *MURDIN AD*	
DNA encoding a 60 kDa cysteine-rich membrane protein.	**WO200055326 -A1**
Patent Inventors *DUNN P* *OOMEN RP* *WANG J* *MURDIN AD*	
A 9 kDa cysteine-rich membrane protein.	**WO200053764 -A1**
Patent Inventors *DUNN P* *OOMEN RP* *WANG J* *MURDIN AD*	
A 60 kDa cysteine-rich membrane protein of Chlamydia pneumoniae.	**WO200055326 -A1**
Patent Inventors *DUNN P* *OOMEN RP* *WANG J* *MURDIN AD*	
C. pneumoniae mip (outer membrane protein).	**WO200006741 -A1**
Patent Inventors *DUNN PL* *OOMEN RP* *MURDIN AD*	
C. pneumoniae mip (outer membrane protein) truncated protein.	**WO200006741 -A1**
Patent Inventors *DUNN PL* *OOMEN RP* *MURDIN AD*	
C. pneumoniae omp protein sequence.	**WO200006743 -A2**
Patent Inventors *DUNN PL* *OOMEN RP* *MURDIN AD*	
C. pneumoniae omp protein truncated sequence.	**WO200006743 -A2**
Patent Inventors *DUNN PL* *OOMEN RP* *MURDIN AD*	
Amino acid sequence of the CPN100111 polypeptide.	**WO200011183 -A2**
Patent Inventors *OOMEN RP* *MURDIN AD*	
Amino acid sequence of the CPN100224 polypeptide.	**WO200011183 -A2**

Patent Inventors *OOMEN RP* *MURDIN AD* **Amino acid sequence of the CPN100230 polypeptide.**	**WO200011183 -A2**
Patent Inventors *OOMEN RP* *MURDIN AD* **Amino acid sequence of the CPN100231 polypeptide.**	**WO200011183 -A2**
Patent Inventors *OOMEN RP* *MURDIN AD* **Amino acid sequence of the CPN100232 polypeptide.**	**WO200011183 -A2**
Patent Inventors *OOMEN RP* *MURDIN AD* **Amino acid sequence of the CPN100233 polypeptide.**	**WO200011183 -A2**
Patent Inventors *OOMEN RP* *MURDIN AD* **Amino acid sequence of the CPN100394 polypeptide.**	**WO200011183 -A2**
Patent Inventors *OOMEN RP* *MURDIN AD* **Amino acid sequence of the CPN100395 polypeptide.**	**WO200011183 -A2**
Patent Inventors *OOMEN RP* *MURDIN AD* **Amino acid sequence of the POMP91A protein of Chlamydia pneumoniae.**	**WO200011180 -A1**
Patent Inventors *DUNN PL* *OOMEN RP* *MURDIN AD* **Chlamydia pneumoniae antigen CPN100202 protein sequence.**	**WO200006739 -A2**
Patent Inventors *OOMEN RP* *MURDIN AD* **Chlamydia pneumoniae antigen CPN100149 protein SEQ ID NO:2.**	**WO200006740 -A1**
Patent Inventors *OOMEN RP* *MURDIN AD* **Chlamydia pneumoniae antigen CPN100605 protein SEQ ID NO:2.**	**WO200006742 -A2**
Patent Inventors *OOMEN RP* *MURDIN AD* **Chlamydia antigen CPN100634.**	**WO200032794 -A2**
Patent Inventors *OOMEN RP* *WANG J* *MURDIN AD* **Chlamydia antigen CPN100635.**	**WO200032794 -A2**
Patent Inventors *OOMEN RP* *WANG J* *MURDIN AD* **Mature Chlamydia antigen CPN100635.**	**WO200032794 -A2**
Patent Inventors *OOMEN RP* *WANG J* *MURDIN AD* **Chlamydia antigen CPN100638.**	**WO200032794**

-A2

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Chlamydia antigen CPN100639. WO200032794 -A2

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Chlamydia antigen CPN100708. WO200032794 -A2

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C. pneumoniae ATP/ADP translocase protein sequence. WO200039157 -A1

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MURDIN AD

Chlamydia pneumoniae 98kD putative outer membrane protein. WO200026237 -A2

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Chlamydia POMP91B precursor protein. WO200026239 -A2

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Chlamydia pneumoniae 98 kDa outer membrane protein CPN100640. WO200032784 -A1

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Chlamydia pneumoniae processed 98 kDa outer membrane protein CPN100640. WO200032784 -A1

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C. pneumoniae mip (outer membrane protein) encoding DNA. WO200006741 -A1

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C. pneumoniae omp protein encoding DNA. WO200006743 -A2

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DNA encoding the CPN100111 polypeptide. WO200011183 -A2

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DNA encoding the CPN100224 polypeptide. WO200011183 -A2

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DNA encoding the CPN100230 polypeptide.	**WO200011183 -A2**
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DNA encoding the CPN100231 polypeptide.	**WO200011183 -A2**
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DNA encoding the CPN100232 polypeptide.	**WO200011183 -A2**
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DNA encoding the CPN100233 polypeptide.	**WO200011183 -A2**
Patent Inventors *OOMEN RP*	
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DNA encoding the CPN100394 polypeptide.	**WO200011183 -A2**
Patent Inventors *OOMEN RP*	
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DNA encoding the CPN100395 polypeptide.	**WO200011183 -A2**
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Nucleotide sequence of the POMP91A gene of Chlamydia pneumoniae.	**WO200011180 -A1**
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Chlamydia pneumoniae antigen CPN100202 nucleotide sequence.	**WO200006739 -A2**
Patent Inventors *OOMEN RP*	
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Chlamydia pneumoniae antigen CPN100149 protein encoding DNA SEQ ID NO:1.	**WO200006740 -A1**
Patent Inventors *OOMEN RP*	
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Chlamydia pneumoniae antigen CPN100605 protein encoding DNA SEQ ID NO:1.	**WO200006742 -A2**
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When such blebs are present in a vaccine formulation they may be more protective against *Chlamydia pneumoniae* infection than the use of the protein/antigen in isolation.

Particularly beneficial pairs of *Chlamydia pneumoniae* antigens have also been found. Thus in a further aspect a Gram-negative bleb (preferably from meningococcus) is provided presenting on its surface both the PorB and MOMP outer membrane proteins from *Chlamydia pneumoniae*. Furthermore, a Gram-negative bleb (preferably from meningococcus) is provided presenting on its surface both MOMP

and one or more Pmp outer membrane proteins from *Chlamydia pneumoniae*. A Gram-negative bleb (preferably from meningococcus) is additionally provided presenting on its surface both the PorB and one or more Pmp outer membrane proteins from *Chlamydia pneumoniae*. A Gram-negative bleb (preferably from meningococcus) is also provided presenting on its surface both the PorB and Npt1 proteins from *Chlamydia pneumoniae*. A Gram-negative bleb (preferably from meningococcus) is additionally provided presenting on its surface both the Npt1 and one or more Pmp proteins from *Chlamydia pneumoniae*. Lastly, a Gram-negative bleb (preferably from meningococcus) is provided presenting on its surface both the Npt1 and MOMP proteins from *Chlamydia pneumoniae*. Bacterial strains from which these blebs are derived are further aspects of this invention.

Such prepartions formulated as a vaccine can give enhanced protection to a host against *Chlamydia* than when a single antigen is administered.

Preferably the bleb has been derived from a strain which has been modified to upregulate one or more protective outer membrane antigens (see below; for instance for meningocococcal protective outer membrane antigens see section "Neisserial bleb preparations" for those antigens that should preferably be upregulated).

Preferably the bleb has been derived from a strain which has been modified to downregulate one or more immunodominant variable or non-protective outer membrane antigens (as described below; for instance for meningocococcal variable/non-protective outer membrane antigens see section "Neisserial bleb preparations" for those antigens that should preferably be downregulated).

Preferably the blebs are derived from a strain which has a detoxified lipid A portion of bacterial LPS, due to the strain having been engineered to reduce or switch off expression of one or more genes which cause LPS to be toxic (preferably selected from the following genes, or homologues thereof htrB, msbB and lpxK; see section below).

Preferably the blebs are derived from a strain which has a detoxified lipid A portion of bacterial LPS, due to the strain having been engineered to express at a higher level of one or more genes producing a gene product that is capable of detoxifying LPS (preferably selected from the following genes, or homologues thereof: pmrA, pmrB, pmrE and pmrF; see section below).

Vaccine compositions comprising the bleb of the invention and a pharmaceutically suitable excipient or carrier are also envisaged. Preferably the vaccine additionally comprising a mucosal adjuvant. Mucosal adjuvants are well known in the art (see Vaccine Design "The subunit and adjuvant approach" (eds Powell M.F. & Newman M.J.) (1995) Plenum Press New York). A preferred mucosal adjuvant is LT2 (or LTII, which can be split into LTIIa and LTIIb – see Martin et al. Infection and Immunity, 2000, 68:281-287). Preferably such vaccines should be formulated and administered as described below in "Vaccine formulations".

The content of blebs per dose in the vaccine will typically be in the range 1-100μg, preferably 5-50μg, most typically in the range 5 - 25μg.

Optimal amounts of components for a particular vaccine can be ascertained by standard studies involving observation of appropriate immune responses in subjects. Following an initial vaccination, subjects may receive one or several booster immunisations adequately spaced.

The efficacy of a *C. pneumoniae* vaccine can be evaluated in a mouse model of infection such as the one described by Murdin et al., 2000, J. Infect. Dis. 181 (suppl 3):S5444-51. The protection elicited by a vaccine formulation can be assessed by reduction of the bacterial load in the lung after a challenge infection with *C. pneumoniae*.

A method of preventing *Chlamydia pneumoniae* infection in a host is also provided comprising the steps of administering an effective amount of the above vaccine to a host in need thereof. Preferably the vaccine is mucosally administered via either an intranasal, intradermal or oral route.

Further improvements in the bacteria and blebs of the invention

The Gram-negative bacterium of the invention may be further genetically engineered by one or more processes selected from the following group: (a) a process of down-regulating expression of immunodominant variable or non-protective antigens, (b) a process of upregulating expression of protective OMP antigens, (c) a process of down-regulating a gene involved in rendering the lipid A portion of LPS toxic, (d) a process of upregulating a gene involved in rendering the lipid A portion of LPS less toxic, and (e) a process of down-regulating synthesis of an antigen which

shares a structural similarity with a human structure and may be capable of inducing an auto-immune response in humans. These processes are described in detail in WO 01/09350 (incorporated by reference herein).

Such bleb vaccines of the invention are designed to focus the immune response on a few protective (preferably conserved) antigens or epitopes - formulated in a multiple component vaccine. Where such antigens are integral OMPs, the outer membrane vesicles of bleb vaccines will ensure their proper folding. This invention provides methods to optimize the OMP and LPS composition of OMV (bleb) vaccines by deleting immunodominant variable as well as non protective OMPs, by creating conserved OMPs by deletion of variable regions, by upregulating expression of protective OMPs, and by eliminating control mechanisms for expression (such as iron restriction) of protective OMPs. In addition the invention provides for the reduction in toxicity of lipid A by modification of the lipid portion or by changing the phosphoryl composition whilst retaining its adjuvant activity or by masking it. Each of these new methods of improvement individually improve the bleb vaccine, however a combination of one or more of these methods work in conjunction so as to produce an optimised engineered bleb vaccine which is immuno-protective and non-toxic - particularly suitable for paediatric use.

(a) a process of down-regulating expression of immunodominant variable or non-protective antigens

Many surface antigens are variable among bacterial strains and as a consequence are protective only against a limited set of closely related strains. An aspect of this invention covers the reduction in expression, or, preferably, the deletion of the gene(s) encoding variable surface protein(s) which results in a bacterial strain producing blebs which, when administered in a vaccine, have a stronger potential for cross-reactivity against various strains due to a higher influence exerted by conserved proteins (retained on the outer membranes) on the vaccinee's immune system. Examples of such variable antigens include: for *Neisseria* - pili (PilC) which undergoes antigenic variations, PorA, Opa, TbpB, FrpB; for *H. influenzae* - P2, P5, pilin, IgA1-protease; and for *Moraxella* - CopB, OMP106.

Other types of gene that could be down-regulated or switched off are genes which, *in vivo*, can easily be switched on (expressed) or off by the bacterium. As outer membrane proteins encoded by such genes are not always present on the bacteria, the presence of such proteins in the bleb preparations can also be detrimental to the effectiveness of the vaccine for the reasons stated above. A preferred example to down-regulate or delete is *Neisseria* Opc protein. Anti-Opc immunity induced by an Opc containing bleb vaccine would only have limited protective capacity as the infecting organism could easily become Opc$^-$. *H. influenzae* HgpA and HgpB are other examples of such proteins.

In process a), these variable or non-protective genes are down-regulated in expression, or terminally switched off. This has the surprising advantage of concentrating the immune system on better antigens that are present in low amounts on the outer surface of blebs.

The strain can be engineered in this way by a number of strategies including transposon insertion to disrupt the coding region or promoter region of the gene, or point mutations or deletions to achieve a similar result. Homologous recombination may also be used to delete a gene from a chromosome (where sequence X comprises part (preferably all) of the coding sequence of the gene of interest). It may additionally be used to change its strong promoter for a weaker (or no) promoter. All these techniques are described in WO 01/09350 (published by WIPO on 8/2/01 and incorporated by reference herein).

(b) a process of upregulating expression of protective OMP antigens

This may be done by inserting a copy of such a protective OMP into the genome (preferably by homologous recombination), or by upregulating expression of the native gene by replacing the native promoter for a stronger promoter, or inserting a strong promoter upstream of the gene in question (also by homologous recombination). Such methods can be accomplished using the techniques described in WO 01/09350 (published by WIPO on 8/2/01 and incorporated by reference herein).

Such methods are particularly useful for enhancing the production of immunologically relevant Bleb components such as outer-membrane proteins and lipoproteins (preferably conserved OMPs, usually present in blebs at low concentrations).

(c) a process of down-regulating a gene involved in rendering the lipid A portion of LPS toxic

The toxicity of bleb vaccines presents one of the largest problems in the use of blebs in vaccines. A further aspect of the invention relates to methods of genetically detoxifying the LPS present in Blebs. Lipid A is the primary component of LPS responsible for cell activation. Many mutations in genes involved in this pathway lead to essential phenotypes. However, mutations in the genes responsible for the terminal modifications steps lead to temperature-sensitive (*htrB*) or permissive (*msbB*) phenotypes. Mutations resulting in a decreased (or no) expression of these genes result in altered toxic activity of lipid A. Indeed, the non-lauroylated (htrB mutant) [also defined by the resulting LPS lacking both secondary acyl chains] or non-myristoylated (msbB mutant) [also defined by the resulting LPS lacking only a single secondary acyl chain] lipid A are less toxic than the wild-type lipid A. Mutations in the lipid A 4'-kinase encoding gene (*lpxK*) also decreases the toxic activity of lipid A.

Process c) thus involves either the deletion of part (or preferably all) of one or more of the above open reading frames or promoters. Alternatively, the promoters could be replaced with weaker promoters. Preferably the homologous recombination techniques are used to carry out the process. Preferably the methods described in WO 01/09350 (published by WIPO on 8/2/01 and incorporated by reference herein) are used. The sequences of the htrB and msbB genes from *Neisseria meningitidis* B, *Moraxella catarrhalis*, and *Haemophilus influenzae* are provided in WO 01/09350 for this purpose.

(d) a process of upregulating a gene involved in rendering the lipid A portion of LPS less toxic

LPS toxic activity could also be altered by introducing mutations in genes/loci involved in polymyxin B resistance (such resistance has been correlated with addition of aminoarabinose on the 4' phosphate of lipid A). These genes/loci could be *pmrE* that encodes a UDP-glucose dehydrogenase, or a region of antimicrobial peptide-resistance genes common to many enterobacteriaciae which could be involved in aminoarabinose synthesis and transfer. The gene *pmrF* that is present in this region

encodes a dolicol-phosphate manosyl transferase (Gunn J.S., Kheng, B.L., Krueger J., Kim K.,Guo L., Hackett M., Miller S.I. 1998. *Mol. Microbiol.* 27: 1171-1182).

Mutations in the PhoP-PhoQ regulatory system, which is a phospho-relay two component regulatory system (f. i. PhoP constitutive phenotype, $PhoP^c$), or low Mg^{++} environmental or culture conditions (that activate the PhoP-PhoQ regulatory system) lead to the addition of aminoarabinose on the 4'-phosphate and 2-hydroxymyristate replacing myristate (hydroxylation of myristate). This modified lipid A displays reduced ability to stimulate E-selectin expression by human endothelial cells and TNF-α secretion from human monocytes.

Process d) involves the upregulation of these genes using a strategy as described in WO 01/09350 (published by WIPO on 8/2/01 and incorporated by reference herein).

(e) a process of down-regulating synthesis of an antigen which shares a structural similarity with a human structure and may be capable of inducing an auto-immune response in humans

The isolation of bacterial outer-membrane blebs from encapsulated Gram-negative bacteria often results in the co-purification of capsular polysaccharide. In some cases, this "contaminant" material may prove useful since polysaccharide may enhance the immune response conferred by other bleb components. In other cases however, the presence of contaminating polysaccharide material in bacterial bleb preparations may prove detrimental to the use of the blebs in a vaccine. For instance, it has been shown at least in the case of *N. meningitidis* that the serogroup B capsular polysaccharide does not confer protective immunity and is susceptible to induce an adverse auto-immune response in humans. Consequently, process e) of the invention is the engineering of the bacterial strain for bleb production such that it is free of capsular polysaccharide. The blebs will then be suitable for use in humans. A particularly preferred example of such a bleb preparation is one from *N. meningitidis* serogroup B devoid of capsular polysaccharide.

This may be achieved by using modified bleb production strains in which the genes necessary for capsular biosynthesis and/or export have been impaired as described in WO 01/09350 (published by WIPO on 8/2/01 and incorporated by reference herein). A preferred method is the deletion of some or all of the *Neisseria*

meningitidis cps genes required for polysaccharide biosynthesis and export. For this purpose, the replacement plasmid pMF121 (described in Frosh et al.1990, *Mol. Microbiol.* 4:1215-1218) can be used to deliver a mutation deleting the *cpsCAD* (+ *galE*) gene cluster. Alternatively the *siaD* gene could be deleted, or down-regulated in expression (the meningococcal *siaD* gene encodes alpha-2,3-sialyltransferase, an enzyme required for capsular polysaccharide and LOS synthesis). Such mutations may also remove host-similar structures on the saccharide portion of the LPS of the bacteria.

Combinations of methods a) – e)

It may be appreciated that one or more of the above processes may be used to produce a modified strain from which to make improved bleb preparations of the invention. Preferably one such process is used, more preferably two or more (2, 3, 4, or 5) of the processes are used in order to manufacture the bleb vaccine. As each additional method is used in the manufacture of the bleb vaccine, each improvement works in conjunction with the other methods used in order to make an optimised engineered bleb preparation.

A preferred meningococcal (particularly *N. meningitidis* B) bleb preparation comprises the use of processes b), c) and e) (optionally combined with process a)). Such bleb preparations are safe (no structures similar to host structures), non-toxic, and structured such that the host immune response will be focused on high levels of protective (and preferably conserved) antigens. All the above elements work together in order to provide an optimised bleb vaccine.

Similarly for *M. catarrhalis*, non-typeable *H. influenzae*, gonococcus, and non serotype B meningococcal strains (e.g. serotype A, C, Y or W), preferred bleb preparations comprise the use of processes b) and c), optionally combined with process a).

Preferred Neisserial bleb preparations

One or more of the following genes (encoding protective antigens) are preferred for upregulation via process b) when carried out on a Neisserial strain, including gonococcus, and meningococcus (particularly *N. meningitidis* B): NspA (WO 96/29412), Hsf-like (WO 99/31132), Hap (PCT/EP99/02766), PorA, PorB,

OMP85 (WO 00/23595), PilQ (PCT/EP99/03603), PldA (PCT/EP99/06718), FrpB (WO 96/31618), TbpA (US 5,912,336), TbpB, FrpA/FrpC (WO 92/01460), LbpA/LbpB (PCT/EP98/05117), FhaB (WO 98/02547), HasR (PCT/EP99/05989), lipo02 (PCT/EP99/08315), Tbp2 (WO 99/57280), MltA (WO 99/57280), and ctrA (PCT/EP00/00135). They are also preferred as genes which may be heterologously introduced into other Gram-negative bacteria.

One or more of the following genes are preferred for downregulation via process a): PorA, PorB, PilC, TbpA, TbpB, LbpA, LbpB, Opa, and Opc (most preferably PorA).

One or more of the following genes are preferred for downregulation via process c): htrB, msbB and lpxK (most preferably msbB which removes only a single secondary acyl chain from the LPS molecule).

One or more of the following genes are preferred for upregulation via process d): pmrA, pmrB, pmrE, and pmrF.

One or more of the following genes are preferred for downregulation via process e): galE, siaA, siaB, siaC, siaD, ctrA, ctrB, ctrC, and ctrD (the genes are described in described in WO 01/09350 - published by WIPO on 8/2/01 and incorporated by reference herein).

Many of the above open reading frames and upstream regions are described in WO 01/09350 (incorporated by reference herein).

Preferred gonococcal genes to upregulate via process b) include one or more of the following:

```
Neisseria gonorrheae lactoferrin receptor precursor (lbpA) gene,
          complete cds.
ACCESSION   U16260
VERSION     U16260.1  GI:915277
Source: Neisseria gonorrhoeae/strain="FA19"
gene="lbpA" nucleotides: 278..3109
protein_id="AAC13780.1"/db_xref="GI:915278"

/translation="MNKKHGFPLTLTALAIATAFPAYAAQAGAAALDAAQSQSLKEVT

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SSDHGSGALGGAVAFRTKEAADLISDGKSWGIQAKTAYGSKNRQFMKSLGAGFSKDGW

EGLLIRTERQGRETRPHGDIADGVEYGIDRLDAFRQTYDIKRKTTEPFFLVEGENTLK

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KPKSVSNRPDLSLRSYALDAVQPSRYVLGFGYDQPEGKWGANIMLTYSKGKNPDELAY

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Neisseria gonorrhoeae lactoferrin binding protein B precursor.
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Neisseria gonorrhoeae strain UU1008 transferrin-binding protein 2 (tbpB) gene, complete cds.

ACCESSION U65222

VERSION U65222.1 GI:2286066

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Neisseria gonorrhoeae pilus biogenesis gene cluster, pilO, pilP and pilQ genes, complete cds.

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NspA
Neisseria gonorrhoeae outer membrane protein gene, complete cds
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VERSION U52069.1 GI:1808968
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    361 catccaccga tttcaaactt tacagcatcg gcgcgtccgt catttacgac ttcgacaccc
    421 aatcgcccgt caaaccgtat ttcggcgcgc gcttgagcct caaccgcgct tccgcccact
    481 tgggcggcag cgacagcttc agcaaaacct ccgccggcct cggcgtattg gcgggcgtaa
    541 gctatgccgt taccccgaat gtcgatttgg atgccggcta ccgctacaac tacgtcggca
    601 aagtcaacac tgtcaaaaac gtccgttccg gcgaactgtc cgccggcgtg cgcgtcaaat
    661 tctgatatac gcgttattcc gcaaaccgcc gagccttcgg cggttttttg
```

Neisseria gonorrhoeae outer membrane protein (omp85) gene, complete cds.
ACCESSION U81959
VERSION U81959.1 GI:1766041

Source: Neisseria gonorrhoeae/strain="FA19"
gene="omp85" coding sequence 1..2379
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/db_xref="GI:1766042"

/translation="MKLKQIASALMMLGISPLAFADFTIQDIRVEGLQRTEPSTVFNY

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MLQNDAIKKNLESFGLAQSQYFNQATLNQAVAGLKEEYLGRGKLNIQITPKVTKLARN

RVDIDITIDEGKSAKITDIEFEGNQVYSDRKLMRQMSLTEGGIWTWLTRSDRFDRQKF

AQDMEKVTDFYQNNGYFDFRILDTDIQTNEDKTRQTIKITVHEGGRFRWGKVSIEGDT

NEVPKAELEKLLTMKPGKWYERQQMTAVLGEIQNRMGSAGYAYSEISVQPLPNAGTKT

VDFVLHIEPGRKIYVNEIHITGNNKTRDEVVRRELRQMESAPYDTSKLQRSKERVELL

GYFDNVQFDAVPLAGTPDKVDLNMSLTERSTGSLDLSAGWVQDTGLVMSAGVSQDNLF

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TTAGGGVRMGIPVTEYDRVNFGLAAEHLTVNTYNKAPKRYADFIKQYGKTDGADGSFK

GLLYKGTVGWGRNKTDSALWPTRGYLTGVNAEIALPGSKLQYYSATHNQTWFFPLSKT

FTLMLGGEVGIAGGYGRTKEIPFFENFYGGGLGSVRGYESGTLGPKVYDEYGEKISYG

GNKKANVSAELLFPMPGAKDARTVRLSLFADAGSVWDGRTYTAAENGNNKSVYSENAH
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```
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1201 gatgccgtcc cgcttgccgg tacgcccgac aaagtcgatt tgaacatgag cctgaccgaa
1261 cgttccaccg gctcgctcga cttgagcgcg ggctgggttc aggataccgg cttggtcatg
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1381 tcgcgaagca aaaccacgct caacggctcg ctgtcgttta ccgacccgta cttcacggca
1441 gacggggtca gcctgggcta cgatatttac ggaaaagcct tcgacccgcg caaagcatcg
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1561 gttaccgaat acgaccgcgt caatttcggg ctggcggcgg aacacctgac cgtcaacacc
1621 tacaacaaag cacccaaacg ctatgccgac tttatcaaac aatacggcaa aaccgacggc
1681 gcagacggca gcttcaaagg cctgctgtac aaaggcactg tcggctgggg gcgcaacaag
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1981 ggctacgaaa gcggcacgct cggcccgaaa gtgtatgacg aatacggcga aaaaatcagc
2041 tacggcggca acaaaaaagc caacgtctcc gccgagctgc tcttcccgat gcccggtgcg
2101 aaagacgcac gcaccgtccg cctgagcctg tttgccgacg caggcagcgt gtgggacggc
2161 agaacctata ccgccgccga aaacggtaac aacaaatcgg tttactcgga aaacgcgcat
2221 aaatccacct ttaccaacga attgcgctat tccgccggcg gcgcggttac ctggctctcg
2281 cctttgggcc cgatgaaatt catctacgcc tacccgctga agaaaaaacc ggaagacgaa
2341 atccaacgct tccaattcca gctcggcacg acgttctaa
```

PldA1 homolog in Neisseria gonorrhoeae
Source: U. of Oklahoma sequencing project
PldA1-like coding sequence:

>GONOCTG01_15 Continuation (15 of 22) of gonoctg01 from base 1400001

```
ATGAATACACGAAATATGCGCTATATTCTTTTGACAGGACTGTTGCCGACGGCATCCGCT
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CACCAGTCCAACGGACAGAGCCGTCCCGAATCGCGTTCGTGGAACAGGATTTATGCCATG
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GGCGTGGTACGCGGATTCCACGGTTACGGCGAGAGCCTGATCGACTACAACCACAAGCAG
AACGGTATCGGTATCGGGTTGATGTTCAACGACTGGGACGGCATCTGA

PldA1-like amino acid sequence
MNTRNMRYILLTGLLPTASAFGETALQCAALTDNVTRLACYDRIFAAQLPSSAGQEGQESKAVLNLTE
TVRSSLDKGEAV
IVVEKGGDALPADSAGETADIYTPLSLMYDLDKNDLRGLLGVREHNPMYLMPFWYNNSPNYAPSSPT
RGTTVQEKFGQQK
RAETKLQVSFKSKIAENLFKTRADLWFGYTQRSDWQIYNQGRKSAPFRNTDYKPEIFLTQPVKADLPFG
GRLRMLGAGFV
HQSNGQSRPESRSWNRIYAMAGMEWGKLTVIPRVWVRAFDQSGDKNDNPDIADYMGYGDVKLQYRL
NDRQNVYSVLRYNP
KTGYGAIEAAYTFPIKGKLKGVVRGFHGYGESLIDYNHKQNGIGIGLMFNDWDGI.

1000 base pairs upstream PldA1-like sequence (usuable for replacing the promoter for a stronger sequence)
>GONOCTG01_15 Continuation (15 of 22) of gonoctg01 from base 1400001
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CGGCGTTTTTTTCGCTGCCGCCGCAGGCTGCCAGAGACAGCGCGGCCAAAACGGCTAATA
CGGATTTTTTCATACGGGCAGATTCCTGATGAAAGAGGTTGGAAAAAAAGAAAACCCCGC
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GCCGCCCGATTTTGCCGTTTTTTTGCGCCGTCAGGGTGTCCGACGGGCGGATAGAGAAAA
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Preferred gonococcal genes to downregulate via process a) include one or more of the following:

```
Neisseria gonorrhoeae iron-regulated outer membrane protein preFrpB
          (frpB) gene, complete cds.
ACCESSION   U13980
VERSION     U13980.1  GI:833694

Source: Neisseria gonorrhoeae/strain="FA19"

gene="frpB" coding sequence: 318..2459
/protein_id="AAC43332.1"
/db_xref="GI:833695"

/translation="MNAPFFRLSLLSLTLAAGFAHAAENNANVALDTVTVKGDRQGSK

IRTNIVTLQQKDESTATDMRELLKEEPSIDFGGGNGTSQFLTLRGMGQNSVDIKVDNA

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```

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QAPSYRETTQSNTNLAYTGKDLGFVEKLDANAYVLEKKRYSADDKDNGYAGNVKGPNH

TRIATRSMNFNFDSRLAEQTLLKYGINYRHQEIKPQAFLNSEFEIKDKEKATNEEKKK

NRENEKIAKAYRLTNPTKTDTGAYIEAIHEIDGFTLTGGLRYDRFKVKTHDGKTVSSS

SLNPSFGVIWQPREHWSFSASHNYAGRSPRLYDALQTHGKRGIISIADGTKAERARNT

EIGFNYNDGTFAANGSYFRQTIKDALANPQNRHDSVAVREAVNAGYIKNHGYELGASY

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      241 attattattt tttcttatcc tgccaaacct taacggtttg gcttaacttc ccttcataca
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     1981 tcaaaaacca cggttacgaa ttgggcgcgt cctaccgcac cggcggcctg accgccaaag
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     2581 cgattcccta aggtgctgaa
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N. gonorrhoeae structural gene for gonococcal protein III (PIII).
ACCESSION X05105
VERSION X05105.1 GI:44889
source:Neisseria gonorrhoeae/db_xref="taxon:485"
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/protein_id="CAA28752.1"
/db_xref="GI:44890"
/db_xref="SWISS-PROT:P07050"

/translation="MTKQLKLSALFVALLASGTAVAGEASVQGYTVSGQSNEIVRNNY

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```

Preferred *Pseudomonas aeruginosa* bleb preparations

One or more of the following genes (encoding protective antigens) are preferred for upregulation via process b): PcrV, OprF, OprI. They are also preferred as genes which may be heterologously introduced into other Gram-negative bacteria.

Preferred *Moraxella catarrhalis* bleb preparations

One or more of the following genes (encoding protective antigens) are preferred for upregulation via process b): OMP106 (WO 97/41731 & WO 96/34960), HasR (PCT/EP99/03824), PilQ (PCT/EP99/03823), OMP85 (PCT/EP00/01468), lipo06 (GB 9917977.2), lipo10 (GB 9918208.1), lipo11 (GB 9918302.2), lipo18 (GB

9918038.2), P6 (PCT/EP99/03038), ompCD, CopB (Helminen ME, et al (1993) Infect. Immun. 61:2003-2010), D15 (PCT/EP99/03822), OmplA1 (PCT/EP99/06781), Hly3 (PCT/EP99/03257), LbpA and LbpB (WO 98/55606), TbpA and TbpB (WO 97/13785 & WO 97/32980), OmpE, UspA1 and UspA2 (WO 93/03761), FhaB (WO 99/58685) and Omp21. They are also preferred as genes which may be heterologously introduced into other Gram-negative bacteria.

One or more of the following genes are preferred for downregulation via process a): CopB, OMP106, OmpB1, TbpA, TbpB, LbpA, and LbpB.

One or more of the following genes are preferred for downregulation via process c): htrB, msbB and lpxK (most preferably msbB).

One or more of the following genes are preferred for upregulation via process d): pmrA, pmrB, pmrE, and pmrF.

Many of the above open reading frames and upstream regions are described in WO 01/09350 (incorporated by reference herein).

Preferred *Haemophilus influenzae* bleb preparations

One or more of the following genes (encoding protective antigens) are preferred for upregulation via process b): D15 (WO 94/12641), P6 (EP 281673), TbpA, TbpB, P2, P5 (WO 94/26304), OMP26 (WO 97/01638), HMW1, HMW2, HMW3, HMW4, Hia, Hsf, Hap, Hin47, Iomp1457 (GB 0025493.8), YtfN (GB 0025488.8), VirG (GB 0026002.6), Iomp1681 (GB 0025998.6), OstA (GB 0025486.2) and Hif (all genes in this operon should be upregulated in order to upregulate pilin). They are also preferred as genes which may be heterologously introduced into other Gram-negative bacteria.

One or more of the following genes are preferred for downregulation via process a): P2, P5, Hif, IgA1-protease, HgpA, HgpB, HMW1, HMW2, Hxu, TbpA, and TbpB.

One or more of the following genes are preferred for downregulation via process c): htrB, msbB and lpxK (most preferably msbB).

One or more of the following genes are preferred for upregulation via process d): pmrA, pmrB, pmrE, and pmrF.

Many of the above open reading frames and upstream regions are described in WO 01/09350 (incorporated by reference herein).

Preparations of membrane vesicles (blebs) of the invention

The manufacture of bleb preparations from any of the aforementioned modified strains may be achieved by harvesting blebs naturally shed by the bacteria, or by any of the methods well known to a skilled person (e.g. as disclosed in EP 301992, US 5,597,572, EP 11243 or US 4,271,147). For Neisseria, the method described in the Example below is preferably used

A preparation of membrane vesicles obtained from the bacterium of the invention is a further aspect of this invention. Preferably, the preparation of membrane vesicles is capable of being filtered through a 0.22 μm membrane.

A sterile (preferably homogeneous) preparation of membrane vesicles obtainable by passing the membrane vesicles from the bacterium of the invention through a 0.22 μm membrane is also envisaged.

Vaccine Formulations

A preferred embodiment of the invention is the formulation of the bleb preparations of the invention in a vaccine which may also comprise a pharmaceutically acceptable excipient.

Vaccine preparation is generally described in Vaccine Design ("The subunit and adjuvant approach" (eds Powell M.F. & Newman M.J.) (1995) Plenum Press New York).

The bleb preparations of the present invention may be adjuvanted in the vaccine formulation of the invention. Suitable adjuvants include an aluminium salt such as aluminum hydroxide gel (alum) or aluminium phosphate, but may also be a salt of calcium (particularly calcium carbonate), iron or zinc, or may be an insoluble suspension of acylated tyrosine, or acylated sugars, cationically or anionically derivatised polysaccharides, or polyphosphazenes.

Suitable Th1 adjuvant systems that may be used include, Monophosphoryl lipid A, particularly 3-de-O-acylated monophosphoryl lipid A, and a combination of monophosphoryl lipid A, preferably 3-de-O-acylated monophosphoryl lipid A (3D-MPL) together with an aluminium salt. An enhanced system involves the combination of a monophosphoryl lipid A and a saponin derivative particularly the combination of QS21 and 3D-MPL as disclosed in WO 94/00153, or a less reactogenic composition

where the QS21 is quenched with cholesterol as disclosed in WO96/33739. A particularly potent adjuvant formulation involving QS21 3D-MPL and tocopherol in an oil in water emulsion is described in WO95/17210 and is a preferred formulation.

The vaccine may comprise a saponin, more preferably QS21. It may also comprise an oil in water emulsion and tocopherol. Unmethylated CpG containing oligo nucleotides (WO 96/02555) are also preferential inducers of a TH1 response and are suitable for use in the present invention.

The vaccine preparation of the present invention may be used to protect or treat a mammal susceptible to infection, by means of administering said vaccine via systemic or mucosal route. These administrations may include injection *via* the intramuscular, intraperitoneal, intradermal or subcutaneous routes; or *via* mucosal administration to the oral/alimentary, respiratory, genitourinary tracts. Thus one aspect of the present invention is a method of immunizing a human host against a disease caused by infection of a gram-negative bacteria, which method comprises administering to the host an immunoprotective dose of the bleb preparation of the present invention.

The amount of antigen in each vaccine dose is selected as an amount which induces an immunoprotective response without significant, adverse side effects in typical vaccinees. Such amount will vary depending upon which specific immunogen is employed and how it is presented. Generally, it is expected that each dose will comprise 1-100μg of protein antigen, preferably 5-50μg, and most typically in the range 5 - 25μg.

An optimal amount for a particular vaccine can be ascertained by standard studies involving observation of appropriate immune responses in subjects. Following an initial vaccination, subjects may receive one or several booster immunisations adequately spaced.

Ghost or Killed Whole cell vaccines

The inventors envisage that the above modified bacterial strains may not only be useful in generating bleb preparations useful in vaccines – they may also be easily used to make ghost or killed whole cell preparations and vaccines (with identical advantages). Methods of making ghost preparations (empty cells with intact envelopes) from Gram-negative strains are well known in the art (see for example WO

92/01791). Methods of killing whole cells to make inactivated cell preparations for use in vaccines are also well known. The terms 'bleb preparations' and 'bleb vaccines' as well as the processes described throughout this document are therefore applicable to the terms 'ghost preparation' and 'ghost vaccine', and 'killed whole cell preparation' and 'killed whole cell vaccine', respectively, for the purposes of this invention.

EXAMPLES

The examples below are carried out using standard techniques, which are well known and routine to those of skill in the art, except where otherwise described in detail. The examples are illustrative, but do not limit the invention.

Example 1: Previously reported examples

Examples describing: Construction of a *Neisseiria meningitidis* serogroup B strain lacking capsular polysaccharides; Construction of versatile gene delivery vectors (the pCMK series) targeting integration in the *porA* locus of *Neisseiria meningitidis*; Construction of a *Neisseiria meningitidis* serogroup B strain lacking both capsular polysaccharides and the major immunodominant antigen PorA; Up-regulation of the NspA outer membrane protein production in blebs derived from a recombinant *Neisseiria meningitidis* serogroup B strain lacking functional *porA* and *cps* genes; Up-regulation of the D15/Omp85 outer membrane protein antigen in blebs derived from a recombinant *Neisseiria meningitidis* serogroup B strain lacking functional *cps* genes but expressing PorA; Construction of versatile promoter delivery vectors; Fermentation processes for producing recombinant blebs; Identification of bacterial promoters suitable for up-regulation antigens-coding genes; Up-regulation of the *N. meningitidis* serogroup B *Omp85* gene by promoter replacement; Up-regulation of the Hsf protein antigen in a recombinant *Neisseiria meningitidis* serogroup B strain lacking functional *cps* genes but expressing PorA; *Expression of the Green Fluorescent Protein in a recombinant Neisseria meningitidis serogroup B strain lacking functional cps genes but expressing PorA;* Up-regulation of the *N. meningitidis* serogroup B *NspA* gene by promoter replacement; Up-regulation of the *N. meningitidis* serogroup B *pldA (omplA)* gene by promoter replacement; Up-regulation of the *N. meningitidis* serogroup B *tbpA* gene by promoter replacement; Up-regulation of the *N. meningitidis* serogroup B *pilQ* gene by promoter replacement; Construction of a *kanR/sacB* cassette for introducing "clean", unmarked mutations in the *N. meningitidis* chromosome; Use of small recombinogenic sequences (43bp) to allow homologous recombination in the chromosome of *Neisseria meningitidis*; Active protection of mice immunized with WT and recombinant *Neisseria meningitidis* blebs; and Immunogenicity of recombinant blebs measured by whole cell

& specific ELISA methods have been described in WO 01/09350 (incorporated by reference herein).

Example 2: Gonococcal blebs expressing *Chlamydia trachomatis* proteins on its surface for use in a vaccine composition

Both *Chlamydia trachomatis* and *N. gonorrhoeae* cause sexually transmitted diseases, including urethritis, cervicitis, salpingitis and pelvic inflammatory disease. Mixed infection with both CT and GC does occur. Therefore, in the design of a vaccine targeting one, or more of these diseases, the possibility to afford protection against both organisms with one single formulation creates a technical advantage.

Protection against N. gono.

A *N. gonorrhoeae* OMV vaccine can be obtained from bleb producing strain(s) in which the expression of one or several genes have been up and/or down regulated. A list of genes encoding *N. gonorrhoeae* proteins for which it is particularly useful to up/down regulate expression is provided above.

A successful vaccine for the prevention of infection by N. gono may require more than one of the following elements: generation of serum and/or mucosal antibodies to facilitate complement mediated killing of the gonococcus, and/or to enhance phagocytosis and microbial killing by leukocytes such as polymorphonuclear leukocytes, and/or to prevent attachment of the gonococci to the host tissues; induction of a cell mediated immune response may also participate to protection.

The potential of a bleb gono vaccine preparation can be evaluated by analyzing the induced immune response for serum and/or mucosal antibodies that have antiadherence, and/or opsonizing properties, and/or bactericidal activity, as described by others (McChesney D et al, Infect. Immun. 36: 1006, 1982; Boslego J et al: Efficacy trialof a purified gonococcl pilus vaccine, in Program and Abstracts of the 24th Interscience Conference on Antimicrobial Agents and Chemotherapy, Whashington, American Society for Microbiology, 1984; Siegel M et al, J. Infect. Dis 145: 300, 1982; de la Pas, Microbiology, 141 (Pt4): 913-20, 1995).

A mouse model of genital infection by N. gono has recently been described (Plante M, J. Infect. Dis., 182: 848-55, 2000). The efficiency of a bleb gono vaccine

could also be evaluated by its ability to prevent or to reduce colonization by N. gono in this mouse model of infection.

Protection against CT

A GC/CT bleb vaccine can be obtained from a strain expressing one or several Chlamydia genes, preferably selected from the above list of genes encoding predicted outer membrane proteins.

Other genes of interest for overexpression in Neisseria are *C. trachomatis* genes for which no homolog has been found in *C. pneumoniae*. Such a set of genes has been described in Richard S. ; p:9-27, Stephens Stephens Ed. ASM Press, Washington DC, Chlamydia: Intracellular Biology, Pathogenesis, and Immunity ISBN: 1-55581-155-8 pages: 380.

Most preferred combinations of *Chlamydia trachomatis* genes are as follows: Major outer membrane protein MOMP (from one or several different serovars) and the Outer membrane Protein Analog (also known as PorB), MOMP (from one or several different serovars) and the Putative Outer Membrane Protein G (pmpG); & PorB and pmpG.

Although the immunity to CT is not fully understood, there is evidence that Ab play a role in protection. Ab to CT in genital fluids have been associated with immunity to CT (Brunham RC, Infect Immun. 1983 Mar;39(3):1491-4.). A protective role of serum antibody in immunity to chlamydial genital infection has also been shown (Rank RG, Infect Immun. 1989 Jan;57(1):299-301.). Antibodies, e.g. MOMP specific antibodies, have been shown to be capable to neutralize CT infection in vitro and in vivo (Caldwell et al. 1982 Infect. Immun. 38: 745-54, Lucero et al, 1985, Infect. Immun. 50: 595-97, Zhang et al. 1987 J. Immunol. 138: 575-581). The MOMP surface antigen of CT has been shown to bear non linear surface epitopes which are target of neutralizing antibodies (Fan J, J. Infect Dis 1997, 176(3):713-21).

Thus, an important objective in the design of a protective chlamydia vaccine includes the identification of formulation(s) of the CT antigens able to optimize the induction of a chlamydia specific antibody responses. Optimization of the Ab response includes targeting to the genital mucosa, and/or presentation of properly folded Chlamydia antigens, and/or combination of several antibody targets.

Mucosal targeting of the immune response to Chlamydia antigen can be achieved by mucosal administration of the vaccine. Intranasal administration of a outer membrane vesicle vaccine can induce persistent local mucosal antibodies and serum antibodies with strong bactericidal activity in humans.

For certain B cell epitopes, such as non linear epitopes, the presentation of the antigen to the immune system in a properly folded manner is critical. A bleb vaccine prepared from a strain expressing Chlamydia antigen(s) offers to chlamydia OMP an outer membrane environment which can be critical to maintain these antigens in a properly folded structure.

Combination of several antibody targets can create an increased efficacy by tackling the infection at different steps of the life cycle of the bacteria, such as adhesion to the host cell, internalization by the host cell and/or interference with further steps of the intracellular development.

The induction and recruitement of Th1 cells into the local genital mucosae are important for immunity against Chlamydia. Thus, an important objective in designing a protective anti-chlamydia vaccine includes the identification of formulation(s) of CT antigen(s) able to optimize the induction of chlamydia speicific Th1 cells, and preferably recruitment of these cells into the genital mucosae. A bleb vaccine prepared from a strain expressing chlamydia antigen(s) can induce a chlamydia specific CMI response. Antigen-specific T-cell responses can be induced in humans after intranasal immunization with an outer membrane vesicle vaccine.

A particular advantage of a GC/CT bleb vaccine is its capability to induce both Ab and CMI responses.

The efficacy of the GC/CT bleb vaccine can be evaluated by its ability to elicit Ag or Chlamydia-specific Ab and/or CMI responses. Ab responses can be evaluated by classical techniques such as ELISA or western blot. Preferably, the induced antibodies can neutralize the infectivity of Chlamydia in an in vitro assay (Byrne G. et al. (J Infect Dis. 1993 Aug;168(2):415-20). Preferably, the CMI response is biased toward the Th1 phenotype. A Th1 biased immune response can be assessed by elevated antigen-specific IgG2a/ IgG1 ratios in mice (Snapper et al. 1987, Science 236:944-47). Elevated ratio of Th1/Th2 cytokine, e.g. elevated IFN-gamma/IL-5, ratio upon in vitro restimulation of immune T cells with the antigen(s) can also indicate such a biased Th1 response.

The ability of the formulation to elicit Ag specific mucosal Ab is of particular interest, and can be demonstrated by detection of antibodies, such as IgG and/or IgA in mucosal fluids, such as genital tract secretions, vaginal lavages. To this end, certain route of administration of the vaccine may be particularly desired such as intranasal, oral, intravaginal, intradermal, deliveries.

The efficacy of the GC/CT bleb vaccine can be evaluated by its ability to induce protection against a Chlamydia challenge in animal model(s). Examples of such animal models have been described in the literature: genital infection with MoPn in mice (Barron et al. J. Infect. Dis. 1143:63-66), genital infection with human strains in mice (Igietseme et al.2000, Infect. Immun. 68:6798-806, Tuffrey et al. 1992 J. Gen. Microbiol. 138: 1707-1715), Tuffrey), genital infection with GPIC strain in guinea pigs (Rank et al. 1992 Am. J. Pathol. 140:927-936). Protection against infection can be assessed by reduction of shed Chlamydia from the infected site and/or reduction of the histopathological reactions after a challenge infection in immunized animals.

The advantage of combining two or more Chlamydia antigens (as described above) can be evaluated by one or more of the following techniques:

- Ability to elicit a multi-target Ab and/or T cell protective response
- Ability to elicit Ab titers in an in vitro neutralizing assay, and/or neutralizing Ab against multiple strains (antigenically distinct)
- Ability to elicit a protective immune response against Chlamydia in a mouse model of genital infection as assessed by reduced shedding of bacteria and/or pathology after challenge.

Example 3: Expression of heterologous antigens (*Clamydia trachomatis* MOMP and PorB) in blebs derived from a recombinant *Neisseiria meningitidis* serogroup B strain lacking functional *porA* and *cps* genes.

Other genes of interest for over-expression in *Neisseria* are *Chlamydia trachomatis* genes for which no homologue has been found in *Chlamydia pneumoniae*. Among those, the major outer membrane protein (MOMP) and the outer membrane protein analog (PorB) have been shown to play a protective role against

chlamydial genital infection. Optimization of the Ab response could be achieved by presentation of properly folded proteins.

MenB bleb vesicles may be used as delivery vectors to express heterologous membrane protein antigens under the control of the engineered *porA-lacO* promoter described in WO 01/09350. Expressed in the bleb context, recombinant MOMP and PorB from *Chlamydia trachomatis* serovar D and K can be correctly folded in the membrane and exposed at the surface. *Neisseiria meningitidis* strains lacking functional *cps* genes are advantageously used as recipient strains to express the heterologous antigens (WO 01/09350).

PCR amplifications of the genes coding for MOMP (*Chlamydia trachomatis).*

Murine McCoy cells (ATCC) infected either, with *Chlamydia trachomatis* Serovar K (UW31-CX-serK), or Serovar D (UW31-CX-serD), were lysed in 400µl of lysis buffer: 50mM KCl, 10mM Tris-HCl pH 8.3, 2.5mM MgCl2, 0.45% Nonidet P40, 0.45% Tween 20 containing 60µg/ml proteinase K, 3 hours at 56°C. Ten µl of the lysate were used as template to amplify the corresponding genes. The gene coding for MOMP (Serovar K) (SEQID N°1 below) was PCR amplified using the CYK/OMP/5/NDE and CYKD/OMP/3/BG oligonucleotide primers (see table 1). The gene coding for MOMP (Serovar D) (SEQID N°2 below) was PCR amplified using the CYD/OMP/5/NRU and CYKD/OMP/3/BG oligonucleotide primers (see table 1). The conditions used for PCR amplification were those described by the supplier (HiFi DNA polymerase, Boehringer Mannheim, GmbH). Thermal cycling was the following: 25 times (94°C 1min., 52°C 1min., 72°C 3min.) and 1 time (72°C 10min., 4°C up to recovery). The corresponding amplicons (1194bp) were digested with either *NdeI/BglII* or *NruI/BglII* restriction enzymes and can be cloned in the corresponding restriction sites of pCMK (+) delivery vector (as described in WO 01/09350).

PCR amplifications of the genes coding for PorB (*Chlamydia trachomatis).*

Murine McCoy cells (ATCC) infected either, with *Chlamydia trachomatis* Serovar K (UW31-CX-serK), or Serovar D (UW31-CX-serD), were lysed in 400µl of lysis buffer: 50mM KCl, 10mM Tris-HCl pH 8.3, 2.5mM MgCl2, 0.45% Nonidet P40, 0.45% Tween 20 containing 60µg/ml proteinase K, 3 hours at 56°C. Ten µl of the lysate were used as template to amplified the corresponding genes.

PorB sequences are highly conserved amongst serovar D and K (SEQID N°3 below). The same primers were used to amplify the corresponding genes in both serovars: CYD/PORB/5/NRU and CYD/PORB/3/BG (see table 1). The conditions used for PCR amplification were those described by the supplier (HiFi DNA polymerase, Boehringer Mannheim, GmbH). Thermal cycling was the following: 25 times (94°C 1min., 52°C 1min., 72°C 3min.) and 1 time (72°C 10min., 4°C up to recovery). The corresponding amplicons (1035bp) were digested with *NruI/BglII* restriction enzymes and can be cloned in the corresponding restriction sites of pCMK (+) delivery vector (as described in WO 01/09350).

Transformation

Linearized recombinant pCMK plasmids can be transformed within a *Neisseria meningitidis* serogroup B strain lacking functional *cps* genes (described in WO 01/09350). Integration resulting from a double crossing-over between the pCMK vectors and the chromosomal *porA* locus can be selected by a combination of PCR and Western Blot screening as described in WO 01/09350.

Table 1: Oligonucleotides used in this work

Oligonucleotides	Sequence	Remark(s)
CYK/OMP/5/NDE	5'-GGG AAT CCA TAT GAA AAA ACT CTT GAA ATC GG-3'	*NdeI* cloning site
CYKD/OMP/3/BG	5'-GGA AGA TCT TTA GAA GCG GAA TTG TGC AT-3'	*Bgl* II cloning site
CYD/OMP/5/NRU	5'-CTG CAG AAT CGC GAA TGA AAA AAC TCT TGA AAT CGG-3'	*NruI* cloning site
CYD/POR/5/NRU	5'-CTG CAG AAT CGC GAA TGA GTA GCA AGC TAG TGA AC-3'	*NruI* cloning site
CYD/POR/3/BG	5'- AGG AGA TCT TTA GAA TTG GAA TCC TCC GG-3'	*Bgl* II cloning site

SEQID N°1:

Nucleotide sequence of DNA coding for *Chlamydia trachomatis* MOMP serovar K protein.

atgaaaaaactcttgaaatcggtattagtatttgccgctttgagttctgcttcctccttgcaagctctgcctgtggggaa
tcctgctgaaccaagccttatgatcgacggaattctgtgggaaggtttcggcggagatccttgcgatccttgcaccactt
ggtgtgacgctatcagcatgcgcgttggttactacggagactttgttttcgaccgtgttttgaaaactgatgtgaataaa
gaatttcagatgggagcggcgcctactaccagcgatgtagaaggcttacaaaacgatccaacaacaaatgttgctcgtcc

aaatcccgcttatggcaaacacatgcaagatgctgaaatgtttacgaacgctgcttacatggcattaaatatctgggatc
gttttgatgtattttgtacattgggagcaactaccggttatttaagaggaaactccgcttccttcaacttagttggatta
ttcggaacaaaaacacaatattctaagtttaatacagcgaatcttgttcctaacactgctttggatcgagctgtggttga
gctttatacagacaccacctttgcttggagcgtaggtgctcgtgcagctctctgggaatgtgggtgtgcaacgttaggag
cttctttccaatatgctcaatctaaacctaaagtagaagagttaaatgttctttgtaatgcatccgaatttactattaat
aagccgaaaggatatgttggggtggaatttccacttgatattaccgcaggaacagaagctgcgacagggactaaggatgc
ctctattgactaccatgagtggcaagcaagtttagccctttcttacagattaaatatgttcactccttacattggagtta
aatggtctagagtaagttttgatgccgacacgatccgtatcgctcagcctaaattggctgaagcaatcttggatgtcact
actctaaacccgaccatcgctggtaaaggagctgtggtctcttccggaagcgataacgaactggctgatacaatgcaaat
cgtttccttgcagttgaacaagctgaaatctagaaaatcttgcggtattgcagtaggaacgactattgtagatgcagata
aatacgcagttacagttgagactcgcttgatcgatgagagagcagctcacgtaaatgcacaattccgcttctaa

SEQID N°2:

Nucleotide sequence of DNA coding for *Chlamydia trachomatis* MOMP serovar D protein.

atgaaaaaactcttgaaatcggtattagtatttgccgctttgagttctgcttcctccttgcaagctctgcctgtggggaa
tcctgctgaaccaagccttatgatcgacggaattctgtgggaaggtttcggcggagatccttgcgatccttgcgccactt
ggtgtgacgctatcagcatgcgtgttggttactacggagactttgttttcgaccgtgttttgaaaactgatgtgaataaa
gaatttcagatgggtgccaagcctacaactgatacaggcaatagtgcagctccatccactcttacagcaagagagaatcc
tgcttacggccgacatatgcaggatgctgagatgtttacaaatgccgcttgcatggcattgaatatttgggatcgttttg
atgtattctgtacattaggagccaccagtggatatcttaaaggaaactctgcttctttcaatttagttggattgtttgga
gataatgaaaatcaaaaaacggtcaaagcggagtctgtaccaaatatgagctttgatcaatctgttgttgagttgtatac
agatactacttttgcgtggagcgtcggcgctcgcgcagctttgtgggaatgtggatgtgcaactttaggagcttcattcc
aatatgctcaatctaaacctaaagtagaagaattaaacgttctctgcaatgcagcagagtttactattaataaacctaaa
gggtatgtaggtaaggagtttcctcttgatcttacagcaggaacagatgctgcgacaggaactaaggatgcctctattga
ttaccatgaatggcaagcaagtttagctctctcttacagactgaatatgttcactccctacattggagttaaatggtctc
gagcaagctttgatgccgatacgattcgtatagcccagccaaaatcagctacagctatttttgatactaccacgcttaac
ccaactattgctggagctggcgatgtgaaaactggcgcagagggtcagctcggagacacaatgcaaatcgtttccttgca
attgaacaagatgaaatctagaaaatcttgcggtattgcagtaggaacaactattgtggatgcagacaaatacgcagtta
cagttgagactcgcttgatcgatgagagagcagctcacgtaaatgcacaattccgcttctaa

SEQID N°3:

Nucleotide sequence of DNA coding for *Chlamydia trachomatis* PorB serovar D protein.

atgagtagcaagctagtgaactatctccgtttgactttcctatctttttagggatcgcatctacttcattagacgctat
gcctgcggggaatccggcgtttccagtcatcccggggattaatattgaacagaaaaatgcctgttctttcgatttatgta
attcttatgatgtactatccgcactgtccggtaacctgaagctctgcttctgcggagattatatcttttcagaagaagct
caggtaaaagatgtccctgtcgttacctctgtgacaacagctggggttggtccttctcctgatattacttcgacaaccaa
aacgcgaaatttcgatctcgtgaactgtaatctcaatacaaactgtgtagctgtagctttttccccttcctgatcgttcgc
tgagcgcgattcctctgtttgatgtgagtttcgaagtgaaagtaggaggactgaaacaatactaccgccttcccatgaat
gcctatcgagacttcacctcggaacctctcaattctgaatcagaagttacggacgggatgattgaagtacagtccaatta
cggatttgtttgggatgttagcttgaaaaaagtcatatggaaagatggcgtttcctttgtaggcgtcggtgcagactatc
gccatgcttcttgccctattgactacatcattgcaaacagtcaagctaatccagaagtattcatcgctgactcggatggg
aaactgaacttcaaggagtggagtgtctgcgtaggtcttactacctatgtgaatgactacgttcttccttacttagcgtt
ttctatagggagtgtttctcgccaagctccggacgacagcttcaaaaaattagaagatcgcttcactaacctcaaattta
aagttcgtaaaattaccagctctcatcgtggaaacatctgcatcggagcgacaaactatgtcgccgataacttcttctac

aacgtagaaggaagatggggaagccagcgcgctgtgaacgtctccggaggattccaattctaa

Example 4: Isolation and purification of blebs from meningococci devoid of capsular polysaccharide

Recombinant blebs can be purified as described below. The cell paste (42gr) is suspended in 211 ml of 0.1M Tris-Cl buffer pH 8.6 containing 10 mM EDTA and 0.5% Sodium Deoxycholate (DOC). The ratio of buffer to biomass should be 5/1 (V/W). The biomass is extracted by magnetic stirring for 30 minutes at room temperature. Total extract is then centrifuged at 20,000g for 30 minutes at 4°C (13,000 rpm in a JA-20 rotor, Beckman J2-HS centrifuge). The pellet should be discarded. The supernatant is ultracentrifuged at 125,000g for 2 hours at 4°C (40,000 rpm in a 50.2Ti rotor, Beckman L8-70M ultracentrifuge) in order to concentrate vesicles. The supernatant should be discarded. The pellet is gently suspended in 25 ml of 50 mM Tris-Cl buffer pH 8.6 containing 2 mM EDTA, 1.2% DOC and 20% sucrose. After a second ultracentrifugation step at 125,000g for 2 hours at 4°C, vesicles are gently suspended in 44 ml of 3% sucrose and stored at 4°C. All solutions used for bleb extraction and purification contained 0.01% thiomersalate. As illustrated in WO 01/019350, this procedure yields protein preparations highly enriched in outer-membrane proteins.

Example 5: Models for testing protection against gonococcal and *C. trachomatis* infection

This can be done as described above in Example 2. In addition Whittum-Hudson et al. (Vaccine 2001 Jul 16;19(28-29):4061-71) "The anti-idiotypic antibody to chlamydial glycolipid exoantigen (GLXA) protects mice against genital infection with a human biovar of Chlamydia trachomatis" is a vaginal inoculation model for C. trachomatis (incorporated by reference herein) which can also be used to test vaccine efficacy.

We Claim:

1. A Gram-negative bacterial bleb presenting on its surface the PorB outer membrane protein from *Chlamydia trachomatis*.

2. The Gram-negative bleb of claim 1 further presenting on its surface the PmpG outer membrane proteins from *Chlamydia trachomatis*.

3. The Gram-negative bleb of claim 1 further presenting on its surface MOMP from one or more serovars from *Chlamydia trachomatis*.

4. A Gram-negative bleb presenting on its surface both the PmpG and MOMP (from one or more serovars) outer membrane proteins from *Chlamydia trachomatis*.

5. The bleb of claims 1-4 which are gonococcal blebs.

6. The bleb of claim 5 which has been derived from a gonococcal strain which has been modified to upregulate one or more protective gonococcal outer membrane antigens.

7. The bleb of claims 5 and 6 derived from a gonococcal strain which has been modified to downregulate one or more immunodominant variable or non-protective gonococcal outer membrane antigens.

8. The bleb of claims 5-7 derived from a strain which has a detoxified lipid A portion of bacterial LPS, due to the strain having been engineered to reduce or switch off expression of one or more genes selected from the group consisting of: htrB, msbB and lpxK.

9. The bleb of claims 5-8 wherein the bleb preparation is derived from a strain which has a detoxified lipid A portion of bacterial LPS, due to the strain having been engineered to express at a higher level one or more genes selected from the group consisting of: pmrA, pmrB, pmrE and pmrF.

10. A vaccine composition comprising the bleb of claims 1-9 and a pharmaceutically suitable excipient or carrier.

11. The vaccine of claim 10, additionally comprising a mucosal adjuvant.

12. A method of preventing *Chlamydia trachomatis* infection in a host comprising the steps of administering an effective amount of the vaccine of claim 10 or 11 to a host in need thereof.

13. The method of claim 12 where in the vaccine is mucosally administered via either a intranasal, oral, or intravaginal route.

14. A Gram-negative bleb presenting on its surface a protective antigen from *Chlamydia pneumoniae.*

15. A Gram-negative bleb presenting on its surface both the PorB and MOMP outer membrane proteins from *Chlamydia pneumoniae.*

16. A Gram-negative bleb presenting on its surface both MOMP and one or more Pmp outer membrane proteins from *Chlamydia pneumoniae.*

17. A Gram-negative bleb presenting on its surface both PorB and one or more Pmp outer membrane proteins from *Chlamydia pneumoniae.*

18. A Gram-negative bleb presenting on its surface both the PorB and Npt1 proteins from *Chlamydia pneumoniae.*

19. A Gram-negative bleb presenting on its surface both Npt1 and one or more Pmp proteins from *Chlamydia pneumoniae.*

20. A Gram-negative bleb presenting on its surface both Npt1 and MOMP proteins from *Chlamydia pneumoniae.*

21. The bleb of claims 14-20 which are meningococcal blebs.

22. The bleb of claim 21 derived from a meningococcal strain that has been modified to upregulate one or more protective meningococcal outer membrane antigens.

23. The bleb of claim 21 or 22 derived from a meningococcal strain that has been modified to downregulate one or more immunodominant variable or non-protective meningococcal outer membrane antigens.

24. The bleb of claims 21-23 derived from a strain which has a detoxified lipid A portion of bacterial LPS, due to the strain having been engineered to reduce or switch off expression of one or more genes selected from the group consisting of: htrB, msbB and lpxK.

25. The bleb of claims 21-24 wherein the bleb preparation is derived from a strain which has a detoxified lipid A portion of bacterial LPS, due to the strain having been engineered to express at a higher level one or more genes selected from the group consisting of: pmrA, pmrB, pmrE and pmrF.

26. A vaccine composition comprising the bleb of claims 14-25 and a pharmaceutically suitable excipient or carrier.

27. The vaccine of claim 26, additionally comprising a mucosal adjuvant.

28. A method of preventing *Chlamydia pneumoniae* infection in a host comprising the steps of administering an effective amount of the vaccine of claim 26 or 27 to a host in need thereof.

29. The method of claim 28 where in the vaccine is mucosally administered via either an intranasal, or oral route.